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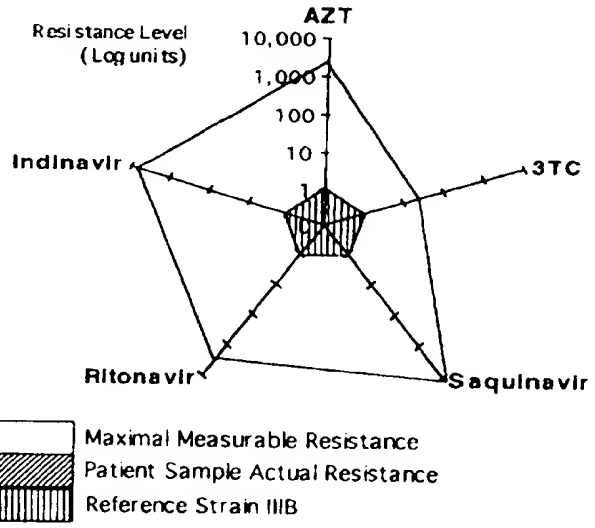
(54) Title: METHOD OF MANAGING THE CHEMOTHERAPY OF PATIENTS WHO ARE HIV POSITIVE BASED ON THE PHENOTYPIC DRUG SENSITIVITY OF HUMAN HIV STRAINS

(57) Abstract

A method of managing HIV chemotherapy of patients who are HIV positive comprises transfecting a cell line susceptible to infection by HIV with a sequence, preferably that coding for RT and protease, from the pol gene of HIV obtained from a patient and a HIV-DNA construct from which the sequence has been deleted, culturing the transfected cells so as to create a stock of chimeric viruses, assessing the phenotypic sensitivity of the chimeric viruses to an inhibitor of the enzyme encoded by the pol gene of HIV and assigning a value thereto, constructing a data set comprising the value for chimeric virus sensitivity and the corresponding value for a chimeric wild-type strain of HIV, repeating the sensitivity assessment for at least two further inhibitors and thereby constructing at least three such data sets in total, representing the data sets in two-dimensional or three-dimensional graphical form such that the difference between the chimeric and wild-type sensitivities in the case of each data set provides a visual measure of the resistance of the chimeric stock to treatment by the inhibitor in question, and selecting the optimum inhibitor(s) on the basis of the graphical representation of the resistances so measured. The method yields phenotypic information on individual HIV infected patients on a large scale, economically and rapidly. The method is applicable to all currently available chemotherapeutic regimens and it is expected to be equally applicable to future chemotherapeutic regimens.



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Description

Method of managing the chemotherapy of patients who are HIV positive based on the phenotypic drug sensitivity of human HIV strains

Technical Field

The present invention relates to a method of managing the chemotherapy of patients who are HIV positive, as well as a clinical management device for use by physicians treating such patients based on the phenotypic drug sensitivity of human HIV strains for inhibitors of one or more enzymes of the pol gene of HIV, as well as a method for simultaneously determining the phenotypic drug sensitivity of two or more of the enzymes of the pol gene of HIV to inhibitors thereof.

Background Art

To date, several chemotherapeutic regimens have been developed for treating HIV infected patients. Certain of these regimens have been approved for clinical use, and others are the subject of on-going clinical trials. It can be assumed that the number of approved chemotherapeutic regimens will increase steadily in the near future. Increasingly, combination therapy or multiple drug treatment regimens are being used because of the development of drug-resistant HIV variants during therapy. Although these chemotherapeutic regimens have been shown to exert an effect on virological (viral load), immunological and clinical parameters of HIV disease, practical experience teaches that these effects are transient. In particular, one finds that the HIV strains infecting an individual patient after a while start to display reduced sensitivity to the drug or drug combination with which said patient is being treated. The loss of efficacy of the chemotherapy can vary from patient to patient, from drug to drug, or from drug combination to drug combination. It is well established that the loss of efficacy to a particular type of chemotherapy can be associated with a genotypic pattern of amino acid changes in the genome of the HIV strains infecting the patient. This probably renders these HIV strains less susceptible to the chemotherapy.

As an HIV infected patient is exposed to several chemotherapeutic regimens over extended periods of time, more complex patterns of amino acid changes in the genome of infecting HIV strains occur which for the present defeat a rational approach to the further treatment of the infected patient. As implied in the previous explanation, one can routinely determine the genotypic changes occurring in HIV strains exposed to different chemotherapeutic regimens involving single or multiple anti-HIV drugs, but thus far it has proven very difficult to derive from these data information enabling a physician in charge of prescribing the chemotherapy whether or not it is sensible to initiate or continue a particular chemotherapeutic regimen. In other words, the genotypic information which is available on a limited scale, cannot routinely be translated into phenotypic information enabling the responsible physician to make the crucial decision as to which chemotherapy a patient should preferably follow. The problem also exists for drug-naive patients who become infected by drug-resistant HIV strains.

Viral load monitoring is becoming a routine aspect of HIV care. However, viral load number alone cannot be used as a basis for deciding which drugs to use alone or in combination.

Combination therapy is becoming increasingly the chemotherapeutic regimen of choice. When a person using a combination of drugs begins to experience drug failure, it is impossible to know with certainty which of the drugs in the combination is no longer active. One cannot simply replace all of the drugs, because of the limited number of drugs currently available. Furthermore, if one replaces an entire chemotherapeutic regimen, one may discard one or more drugs which are active for that particular patient. Furthermore, it is possible for viruses which display resistance to a particular inhibitor to also display varying degrees of cross-resistance to other inhibitors.

Ideally, therefore, every time a person has a viral load test and a viral load increase is detected, a drug sensitivity/resistance test should

also be carried out. Until effective curative therapy is developed, management of HIV disease will require such testing.

Currently there does exist a phenotyping method which is based on virus isolation from plasma in the presence of donor peripheral blood mononuclear cells (PBMCs), and subsequent phenotyping in said cells (Japour, A.J., *et al.* (1993) Antimicrobial Agents and Chemotherapy; Vol. *37*, No. 5, p1095-1101). This co-cultivation method, which is advocated by the AIDS Clinical Trial Group (ACTG) - particularly for phenotyping AZT (synonymous herein with zidovudine/Retrovir (Retrovir is a Trade Mark)) resistance, is time-consuming, costly and too complex to be used on a routine basis.

A phenotypic recombinant virus assay for assessment of drug susceptibility of HIV Type 1 isolates to reverse transcriptase (RT) inhibitors has been developed by Kellam, P. and Larder, B.A. (Antimicrobial Agents and Chemotherapy (1994) Vol. *38*, No. 1, p23-30). This procedure allows the generation of viable virus by homologous recombination of a PCR-derived pool of RT coding sequences into an RT-deleted, noninfectious proviral clone, pHIVΔRTBstEII. Analysis of two patients during the course of zidovudine therapy showed that this approach produced viruses which accurately exhibited the same genotype and phenotype as that of the original infected PBL DNA. However, the procedure involves isolation of the patient virus by co-cultivation of patient plasma or patient PBMCs with donor PBMCs. Such prior cultivation of virus may distort the original virus composition. Furthermore, this method, although allowing one to determine the sensitivity of the isolates to various inhibitors, does not provide the physician with information as to whether to continue with the existing chemotherapeutic regimen or to alter the therapy.

Also when one enzyme only of the pol gene is being studied, the method does not readily lend itself to routine phenotypic assessment of combination therapy which conventionally involves the use of one protease and 2 RT inhibitors.

The nested PCR (polymerase chain reaction) procedure used in the recombinant virus assay can lead to a situation where the recombinant virus does not truly reflect the situation with the HIV strains infecting the patient under investigation. This problem resides in DNA sequence homology and the minimum amount of homology required for homologous recombination in mammalian cells (C. Rubnitz, J. and Subramini, S. (1984) Molecular and Cellular Biology Vol. *4*, No. 11, p2253-2258). Accordingly, any phenotypic assay based on the recombinant virus approach should endeavour to ensure that as much as possible of the patient material is amplified and that there is maximum recombination.

Thus, the RNA extraction and nested PCR procedures employed should ensure that the viral genetic material is amplified such that the amplified material maximally reflects the viral genetic diversity in the patient being investigated.

In current clinical practice there is therefore a hard-felt need (a) to determine rapidly and on a routine basis the phenotypic drug sensitivity of HIV strains infecting a particular patient, (b) to process the thus obtained data into easily understood information, and (c) to initiate, continue or adjust on the basis of said information the chemotherapy prescribed for said particular patients.

Disclosure of the Invention

According to a first aspect of the invention there is provided a method of managing HIV chemotherapy of patients who are HIV positive, which comprises transfecting a cell line susceptible to infection by HIV with a sequence from the pol gene of HIV obtained from a patient and a HIV-DNA construct from which said sequence has been deleted, culturing said transfected cells so as to create a stock of chimeric viruses, assessing the phenotypic sensitivity of said chimeric viruses to an inhibitor of said enzyme encoded by the pol gene of HIV and assigning a value thereto, constructing a data set comprising said value for chimeric virus sensitivity and the corresponding value for a

chimeric wild-type strain of HIV, repeating the sensitivity assessment for at least two further inhibitors and thereby constructing at least three such data sets in total, representing said data sets in two dimensional or three dimensional graphical form such that the difference between the chimeric and wild-type sensitivities in the case of each data set provides a visual measure of the resistance of the chimeric stock to treatment by the inhibitor in question, and selecting the optimum inhibitor(s) on the basis of the graphical representation of the resistances so measured.

The method according to the invention yields phenotypic information on individual HIV infected patients on a large scale, economically and rapidly. The method is applicable to all currently available chemotherapeutic regimens and it is expected to be equally applicable to future chemotherapeutic regimens.

The method according to the invention provides the physician with phenotypic data on patient HIV strains which can be immediately used to determine whether a particular chemotherapeutic regimen should be initiated, continued or adjusted.

Preferably, the data sets are represented on a polygonal or quasi-circular graph comprising:

(a) a plurality of normalised axes extending radially from an origin, each axis corresponding to one data set or inhibitor or combination thereof;

(b) the axes being normalised such that the sensitivity values for wild-type HIV for the various inhibitors are equal on each axis, the data points for wild-type HIV being optionally represented and connected to form a regular polygon whose vertices lie on the axes and whose center is defined by the origin;

(c) on each axis a data point representing the sensitivity value of the chimeric HIV stock against the inhibitor corresponding to said axis is plotted, the chimeric data points being optionally connected to form a regular or irregular polygon the shape of which represents the resistance of the chimeric stock to a range of inhibitors.

A polygonal or quasi-circular graph provides the advantage that the patient's resistance to a number of drugs is characterised in terms of the degree of divergence between the polygon representing the patient's chimeric HIV stock and the polygon representing the wild-type strain. The areas of the polygons will generally diverge more in some areas than in others, indicating in each case a greater or lesser degree of resistance to the inhibitor whose axis passes through the area in question.

Thus, the method according to the invention takes a chimeric HIV stock and provides a map of the resistance of this stock across a range of inhibitors. In this way the map or graph provides a technical characterisation of an aspect of the chimeric stock which is not obtained by conventional measurements.

In a preferred embodiment, the normalised axes are equiangular from one another.

Further, preferably, each axis has a logarithmic scale.

Further, preferably, eccentric data points in the chimeric polygon, if represented, identify inhibitors whose usefulness can be assumed to be of little or no benefit to the patient, while data points lying within, on or close outside the wild-type polygon identify inhibitors whose usefulness can be assumed to be of substantial benefit to the patient.

When worst case values are represented along with the chimeric and wild-type HIV, a usually irregular polygon encloses the chimeric and wild-type polygons. The meaning of the term "eccentric" as used above denotes a data point lying relatively close to the worst-case border

and relatively far from the wild-type polygon. Similarly the term "close outside the wild-type polygon" refers to relative closeness to the wild-type polygon when compared to the distance from the worst-case border.

The method as hereinabove defined is limited in the sense that the measurable resistance against an inhibitor is dependent on the particular range of concentrations of the inhibitor used. Also one must endeavour to reduce the effects of biological variability. Accordingly, it is desirable to obtain a value for maximum or worst-case measurable resistance where it is assumed that a given inhibitor has no effect. This concentration, e.g. 100 μM, is generally the maximum concentration that can practically be tested, but may also be derived from e.g. pharmacological, toxicological or pharmacokinetic studies. The comparison of the resistance level of the patient under investigation and the maximum measurable resistance determines what is the significant level of resistance for the patient under investigation. The maximum measurable resistance and the actual resistance can be suitably shown on a bar graph as hereinafter described.

In a still further preferred embodiment of the invention each of said three or more data sets further comprises a value for worst-case measurable resistance for the inhibitor in question, said worst case values being represented on said graphical representations such that the data point for the chimeric stock can be compared both to wild-type and to worst-case HIV, thereby providing an assessment of the relative value of the inhibitor in a particular case.

Experiments with in excess of 150 patient samples have revealed a close correlation between resistance development and therapy history as hereinafter further illustrated in the Examples. A close correlation has been found with the data generated in accordance with the invention relative to classical virus isolation and phenotyping techniques.

The method in accordance with the invention can thus be used for an individualised and more rational management of HIV chemotherapy. Thus, use of the method according to the invention in combination with

the proper administration of anti-HIV drugs should ultimately lead to better treatment and survival of patients infected with the HIV virus.

The method according to the invention has particular application where an individual patient has been receiving many different drugs and his mutation pattern is not readily interpreted by attending physicians.

According to a further aspect of the invention there is provided a method of managing HIV chemotherapy of patients who are HIV positive, which comprises the steps of:

(a) periodically assessing the phenotypic sensitivity of a patient's HIV strains by a method hereinabove described;

(b) maintaining the chemotherapy with the selected inhibitor while the patient's HIV strains remain susceptible to the selected chemotherapy;

(c) selecting a different inhibitor if and when the susceptibility of the original inhibitor decreases.

According to a still further aspect of the invention there is provided a clinical management device for use in the management of chemotherapy of patients who are HIV positive, said device bearing a graphical representation of a plurality of data sets as hereinabove defined.

We have coined the term "Antivirogram" for the clinical management device according to the invention and this term will be used hereinafter in the specification. This device provides the physician with a clear representation of the relative changes and susceptibilities for different inhibitors which are or which may be used in the clinical management of individual HIV-infected patients.

By HIV herein is generally meant HIV-1. However, the invention is also applicable to HIV-2.

Preferably, the phenotypic sensitivity of said chimeric viruses to inhibitors of at least two enzymes encoded by the pol gene of HIV is simultaneously assessed.

In a further aspect of the invention there is provided a method of determining the phenotypic drug sensitivity of individual HIV strains in a patient to inhibitors of at least two enzymes encoded by the pol gene of HIV, which comprises transfecting a cell line susceptible to infection by HIV with a sequence from the pol gene of HIV obtained from a patient and a HIV-DNA construct from which said sequence has been deleted, culturing said transfected cells so as to create a stock of chimeric viruses and assessing the phenotypic sensitivity of said chimeric viruses to inhibitors of said enzymes encoded by the pol gene of HIV.

The desired sequence from the pol gene is isolated from a sample of a biological material obtained from the patient whose phenotypic drug sensitivity is being determined. A wide variety of biological materials can be used for the isolation of the desired sequence.

Thus, the biological material can be selected from plasma, serum or a cell-free body fluid selected from semen and vaginal fluid. Plasma is particularly preferred and is particularly advantageous relative to the use of PBMCs as used in the prior art described above.

Alternatively, the biological material can be whole blood to which an RNA stabiliser has been added.

In a still further embodiment, the biological material can be a solid tissue material selected from brain tissue or lymph nodal tissue, or other tissue obtained by biopsy.

As hereinafter demonstrated, when a biological material such as plasma is used in the isolation of the desired sequence, a minimal

volume of plasma can be used, typically about 100-250µl, more particularly of the order of 200µl.

Further, preferably the two enzymes selected will be selected from HIV RT, protease and integrase.

Viral RNA is conveniently isolated in accordance with the invention by methods known *per se*, for example the method of Boom, R. *et al.* (Journal of Clinical Microbiology (1990) Vol. *28*, No. 3, p.495-503).

In the case of plasma, serum and cell-free body fluids, one can also use the QIAamp viral RNA kit marketed by the Qiagen group of companies.

Preferably, the cell line susceptible to infection by HIV is a $CD4^+$ T-cell line.

Further, preferably, the $CD4^+$ T-cell line is the MT4 cell line or the HeLa $CD4^+$ cell line.

Reverse transcription can be carried out with a commercial kit such as the GeneAmp Reverse Transcriptase Kit marketed by Perkin Elmer.

The desired region of the patient pol gene is preferably reverse transcribed using a specific downstream primer.

In the case where the sequence to be reverse transcribed is that coding for reverse transcriptase or reverse transcriptase and protease, the downstream primer is preferably OUT3 : 5'-CAT TGC TCT CCA ATT ACT GTG ATA TTT CTC ATG-3' (SEQ ID NO: 1).

In a particularly preferred embodiment a patient's HIV RT gene and HIV protease gene are reverse transcribed using the HIV-1 specific OUT 3 primer and a genetically engineered reverse transcriptase lacking

RNase H activity, such that the total RNA to be transcribed is converted to cDNA without being degraded. Such a genetically engineered reverse transcriptase, the Expand (Expand is a Trade Mark) reverse transcriptase, can be obtained from Boehringer Mannheim GmbH.

Expand reverse transcriptase is a RNA directed DNA polymerase. The enzyme is a genetically engineered version of the Moloney Murine Leukaemia Virus reverse transcriptase (M-MuLV-RT). Point mutation within the RNase H sequence reduces the RNase H activity to below the detectable level. Using this genetically engineered reverse transcriptase enables one to obtain higher amounts of full length cDNA transcripts.

Following reverse transcription the transcribed DNA is amplified using the technique of PCR.

Preferably, the product of reverse transcription is amplified using a nested PCR technique.

Preferably, in the case where the region of interest is the RT region, a nested PCR technique is used using inner and outer primers as described by Kellam, P. and Larder, B.A. (1994 *supra*). When the region of interest is that spanning the RT and protease genes, the specific primers used are preferably a combination of OUT 3/IN 3 (downstream) and RVP 5 (upstream).

The primer RVP 5 (Maschera, B., *et al.* Journal of Virology, *69*, 5431-5436) has the sequence 5'-GGGAAGATCTGGCC TTCCTACAAGGG-3' (SEQ ID NO: 2).

A schematic representation of the amplification is set forth in Fig. 3 and is described in greater detail in Example 2.

The amplification of the protease cDNA actually involves a hemi-nested PCR procedure as will be apparent from Fig. 3.

The nested PCR technique has the advantage over the known simple PCR techniques in that it enables one to obtain the most specific PCR product.

However, to obtain an even higher fidelity and yield during PCR, one can make use of a mixture of thermostable polymerases (Barnes, W.M. (1994) Proc. Natl. Acad. Sci. U.S.A. *91*, 2216-2220). Such a polymerase mixture is available from Boehringer Mannheim GmbH, namely the Expand (Expand is a Trade Mark) high fidelity PCR system. Using this system we have obtained increased sensitivity, namely a sensitivity which is ten times or greater than that obtained with a conventional PCR procedure using Taq polymerase alone.

When the region of the pol gene is that embracing the RT and protease genes, preferably the HIV-DNA construct is one from which the RT and protease genes are deleted and is the plasmid pGEMT3-ΔPRT as deposited at the Belgian Coordinated Collections of Microorganisms-BCCM LMBP-Collection on November 8, 1996 under the number LMBP3590.

However, several approaches can be adopted to generate a plasmid containing the HIV-1 provirus carrying a deletion for the protease as well as for the RT gene. One possibility is the introduction of the desired deletion by means of oligonucleotide-mediated mutagenesis. However, the procedure adopted hereinafter in Example 2 involves the generation of the desired construct by making use of specific restriction enzymes and subcloning procedures, as hereinafter described. Although the final results depend on the available restriction sites a major advantage of this procedure is that one can obtain conclusive results rapidly.

To ensure the most efficient outcome for the transfection, the PCR-product, being transfected, should ideally be purified by anion exchange spin columns in a manner known *per se*. A suitable kit is the QIAquick PCR Purification Kit marketed by the Qiagen group of companies.

Transfection can be achieved by electroporation or, alternatively, by the use of lipids, especially cathionic lipids, DEAE dextran, $CaHPO_4$, etc.

In the case of lipid transfection one can avail of a PERFECT (PERFECT is a Trade Mark) transfection kit marketed by Invitrogen B.V. of Leek, the Netherlands.

Thus, for transfection an HIV-DNA construct from which the gene or genes of choice from the pol gene has/have been deleted is used in conjunction with the product obtained following amplification.

The construct can be the plasmid pHIVΔRT (obtainable from the Medical Research Council (MRC)) if it is the RT gene only that is deleted. When the RT and protease genes are both deleted a suitable HIV DNA construct is the plasmid pGEMT3-ΔPRT described herein and which is a high copy vector. Such plasmids are linearised prior to transfection according to methods known *per se*.

A particular advantage of using a construct coding for more than one pol gene enzyme, for example a ΔPRT construct, is that one is more likely to include more of the original patient material in the construct than if a single gene is used, so that the amplified material reflects to a greater extent the viral genetic diversity in the particular patient being investigated.

It will be appreciated that it is preferable that the specific primers selected for the nested PCR are located outside the body sequences of the target enzymes to be amplified and investigated. It will furthermore be appreciated that a combination of RT and protease is likely to provide better results for studying RT than RT alone, because forty more amino acids are patient borne relative to the situation with RT alone. For studying the protease, one should be aware that the first nine amino acids of the protease are still derived from the construct's (pGEMT3-ΔPRT) wild-type backbone.

When the transfection of the cells is achieved through electroporation, the parameters selected are optimized to achieve good cell growth and virus production. The electroporation can conveniently be conducted at approximately 250μF and 300V. Preferably, the electroporation is conducted in the presence of about 10μg of linearised plasmid e.g. pHIVΔRTBstEII and about 5μg of amplified PCR product e.g. RT PCR product. Upon successful intracellular homologous recombination, new chimeric HIV is formed within 5 to 10 days. With known techniques typical cultivation times are 12-14 days before chimeric HIV is formed. Culture supernatant aliquots are stored at -70°C or lower temperatures.

It is readily seen that one can use the above methods for isolating and amplifying other HIV genes, e.g. the integrase gene, or more than one other HIV gene, e.g. both the RT and the integrase gene, and transfecting a $CD4^+$ T-cell with the respective integrase or RT/integrase PCR products in conjunction with an appropriate linearised HIV-DNA construct from which the relevant gene (or genes) is deleted.

The newly formed chimeric viruses are titrated and then analysed for their phenotypic sensitivity (i.e. susceptibility) to the different pol gene enzyme inhibitors, preferably in an automated cellular-based assay.

Preferably, the phenotypic drug sensitivity of the chimeric viruses and of the wild HIV strain, which is suitably a recombinant wild HIV strain, to one or more RT, protease or integrase inhibitor(s) is expressed as an inhibitory concentration (IC value).

The susceptibilites of the chimeric viruses and of the wild type HIV strain to one or more RT inhibitors and/or one or more protease inhibitors and/or one or more integrase inhibitors can be expressed as for example 50% or 90% inhibitory concentrations (IC_{50} or IC_{90} values).

Preferably, RT inhibitors are selected from nucleoside RT inhibitors such as AZT, ddI (didanosine/Videx (Videx is a Trade Mark), ddC (zalcitabine), 3TC (lamivudine), d4T (stavudine), non-nucleoside

RT inhibitors such as delavirdine (U 9051125 (BMAP)/Rescriptor (Rescriptor is a Trade Mark)), loviride (alpha-APA), nevirapine (B1-RG-587/Viramune (Viramune is a Trade Mark) and tivirapine (8-Cl-TIBO(R86183)), protease inhibitors such as saquinavir, indinavir and ritonavir and integrase inhibitors such as caffeic acid phenylethyl ester (CAPE).

Suitable RT and/or protease inhibitors and/or integrase inhibitors are selected from nucleoside RT inhibitors such as AZT, ddI, ddC, 3TC, d4T, 1592U89 and the like, non-nucleoside RT inhibitors such as loviride, nevirapine, delaviridine, ateviridine, and tivirapine (8-Cl TIBO) and the like, protease inhibitors such as saquinavir, indinavir and ritonavir and the like, and integrase inhibitors such as caffeic acid phenylethyl ester (CAPE) and HIV integrase inhibitors of the type described in WO 95/08540 and GB 2,271,566.

The method according to the invention comprises the step of comparing the phenotypic drug sensitivity of patient HIV strains with one or more RT inhibitors and/or one or more protease inhibitors, and/or one or more integrase inhibitors to that of a wild type HIV strain. For an easy-to-understand representation of the relative changes in susceptibility to the different drug compounds (or combinations) tested, an Antivirogram graph, is constructed.

The graph should be interpreted as follows : eccentric data points in the antivirogram identify chemotherapeutic regimens unlikely to benefit the HIV infected patient any further, whereas data points within or on the reference polygon, or only slightly beyond the reference polygon, identify chemotherapeutic regimens likely to benefit the HIV infected patient.

The methods according to the invention in combination with the administration of the correct anti-HIV drugs should ultimately lead to better treatment, improved quality of life and improved survival of HIV infected patients ; i.e. ineffective treatment (due to the presence of or

emergence of resistant HIV strains) can be prevented or halted, and effective chemotherapy can be initiated in good time.

The present invention also concerns a clinical management device for use by physicians treating HIV infected patients comprising an Antivirogram obtainable by the methods hereinbefore described.

Brief Description of the Drawings

Fig. 1 is a schematic representation of the construction of the plasmid pGEMT3-ΔPRT;

Fig. 2 is a further and complementary schematic representation of the construction of the plasmid pGEMT3-ΔPRT;

Fig. 3 is a schematic representation of that part of the HIV-HXB2D sequence containing protease and RT genes;

Fig. 4A-H is a complete sequence for that part of the HIV-HXB2D sequence containing protease and RT genes;

Fig. 5 is an Antivirogram for a patient harbouring 3TC resistant HIV strains as described in Example 5;

Fig. 6 is an Antivirogram for a drug-naive patient harbouring wild type like HIV strains as described in Example 6;

Fig. 7A is a bar graph showing relative change in drug susceptibility for the patient of Example 7;

Fig. 7B is an Antivirogram for the patient the subject of Example 7;

Fig. 8A is a bar graph showing relative change in drug susceptibility for the patient of Example 8;

Fig. 8B is an Antivirogram for the patient the subject of Example 8;

Fig. 9A is a bar graph showing relative change in drug susceptibility for the patient of Example 9;

Fig. 9B is an Antivirogram for the patient the subject of Example 9;

Fig. 10A is a bar graph showing relative change in drug susceptibility for the patient of Example 10;

Fig. 10B is an Antivirogram for the patient the subject of Example 10;

Fig.11A is a bar graph showing relative change in drug susceptibility for the patient of Example 11;

Fig.11B is an Antivirogram for the patient the subject of Example 11;

Fig.12A is a bar graph showing relative change in drug susceptibility for the patient of Example 12; and

Fig.12B is an Antivirogram for the patient the subject of Example 12.

The invention will be further illustrated by the following Examples.

Modes for Carrying Out the Invention

Example 1

Protocol

1. Extraction and amplification of viral RNA.

RNA was isolated from 100µl of plasma according to the method described by Boom, R. *et al.* (1990, *supra*), and was reverse transcribed with the GeneAmp reverse transcriptase kit (Perkin Elmer) as described by the manufacturer and using an HIV-1 specific downstream primer (OUT3 : 5'-CAT TGC TCT CCA ATT ACT GTG ATA TTT CTC ATC-3'; SEQ ID NO: 1). PCR on reverse transcribed RNA was performed with inner and outer primers as described by Kellam , P. and Larder, B.A. (1994, *supra*). After chloroform extraction and centrifugation on Centricon 100 columns or centrifugation on anion-exchange spin columns (Quiagen), the isolated PCR product was ready for use in the transfection reactions.

2. Production and isolation of plasmid.

Production of pHIVΔRT (MCR) plasmid was performed in *E. coli*. Plasmid DNA was isolated from overnight cultures making use of Qiagen columns as described by the manufacturer. Yield of the isolated plasmid was determined spectrophotometrically by A260/280 measurement (optical density measurement at λ = 260 and 280 nm). About 250 µg of ultrapure plasmid DNA was obtained from 500 ml of bacterial culture. The identity of the isolated plasmid was confirmed by restriction analysis. Subsequently, the isolated plasmid DNA was linearised with BstEII and purified again by a classical phenol/chloroform extraction.

3. Transfection of cells.

MT4 cells were subcultured at a density of 250,000 cells/ml before transfection (exponential growth phase). Cells were pelleted and

resuspended in phosphate buffered saline (PBS) at a concentration of 3.1 10E6 cells/ml. A 0.8 ml portion (2.5 10E6 cells/ml) was used for each transfection. Transfection was performed with the Bio-Rad Gene pulser making use of 0.4 cm electrode cuvettes. Cells were electroporated in the presence of 10µg of linearised pHIVΔRT plasmid and approximately 5µg of RT PCR product at 250µF and 300 V, followed by a 30-min incubation at room temperature. Subsequently, 10 ml of fresh culture medium was added to the cell suspension and incubation was performed at 37°C in a humidified atmosphere of 5% CO_2.

4. Culture and follow-up of transfected cells.

During 7 to 10 days following the transfection, cells were monitored for the appearance of cytopathogenic effect (CPE). In the absence thereof, cells were subcultured in different flasks. Subsequently, culture supernatants of transfected cells were used to create a stock of recombinant virus and stored in 1.5 ml aliquots at -70°C.

5. Analysis of recombinant virus from patient viral RNA.

After titration of the new viruses, the stocks were used for antiviral experiments in the presence of serial dilutions of different HIV inhibitors. Titres of the harvested supernatants were determined by limited serial dilution titration of virus in MT4 cells.

Viruses with a useful titre were used in antiviral experiments. For this purpose, 96-well microtitre plates were filled with 100µl of complete culture medium. Subsequently, stock solutions of compounds were added in 25µl volumes to series of duplicate wells. HIV- and mock-infected cell samples were included for each drug (or drug combination). Exponentially growing MT4 cells were then transferred to the microtitre plates at a density of 150,000 cells/ml. The cell cultures were then incubated at 37°C in a humidified atmosphere of 5% CO_2. Five days after infection, the viability of the mock- and HIV-infected cells was examined spectrophotometrically by the MTT method (Pauwels, R. *et al.* - J. Virol. Meth. (1988), *20* : 309-321) as described in Section 6 below.

6. MTT assay.

To each well of the microtiter plates, 20µl of a solution of MTT (7.5 mg/ml in PBS) was added. The plates were further incubated at 37°C for 1 h. Then, 150µl of medium was removed without disturbing the MT4 cell clusters containing the formazan crystals. Solubilization of the formazan crystals was achieved by adding 100µl 5% Triton X-100 in acidified isopropanol (5 ml concentrated HCl per litre solvent). Complete dissolution of the formazan crystals was obtained after the plates had been placed on a plate shaker for 10 min. Finally the absorbances were read at two wavelengths (540 and 690 nm). From these optical density (OD) data, 50% inhibitory (IC_{50}) and 50% cytotoxic (CC_{50}) concentrations were derived.

Example 2

Construction of a pHIVΔRTBstEII-variant with deletion of the HIV-1 protease and reverse transcriptase gene.

The protocol described in Example 1 was repeated, except that the sequence of the HIV pol gene of interest was that coding for RT and protease and the construct prepared was pGEMT3-ΔPRT as described below. Other modifications relative to the procedure set out in Example 1 are set out below.

For amplification of viral RNA, reverse transcription from RNA to DNA was again carried out with the OUT3 primer. However, for the nested PCR procedure the primers used are as shown in Fig. 3. Thus, it will be observed that the nested PCR procedure uses as outer primers RVP5 and OUT3 and as inner primers RVP5 and IN3. Thus, this nested procedure is, in effect a hemi-nested PCR procedure.

Production and isolation of pGEMT3-ΔPRT.

The final pGEMT3-ΔPRT construct is a derivative of pGEM9-Zf(-) (Promega).

In short, the pGEMT3-ΔPRT construct is built up by introducing the desired insert HIV-HXB2 (a protease and reverse transcriptase-deleted proviral HIV-1 clone, including flanking human sequences) into the XbaI restriction site of the vector pGEM9-Zf(-). The proviral genome has been deleted from the AhdI site within the protease gene (surrounding amino acid 9) to the BstEII site of the pHIVΔRTBstEII construct (MRC Repository reference : ADB231). At the junction of the ΔProRT deletion SmaI and BstEII sites are located which can be used for linearisation of the proviral construct prior to transfection. The construction of pGEMT3-ΔPRT is schematically represented in Figs 1 and 2. The yield of pGEMT3-ΔPRT was about 1mg out of 500ml bacterial culture.

As indicated above, the plasmid pGEMT3-ΔPRT was deposited at the Belgian Coordinated Collections of Microorganisms-BCCM LMBP-Collection on November 8, 1996 under the number LMBP3590.

It was not expected that the introduction of the proviral genome into another vector (pGEM9-Zf(-) instead of pIB120) would cause major problems. pIB120, a derivative of pEMBL8(-) (according to information provided by Kodak Scientific Imaging Systems), and pGEM9-ZF(-) are similar vectors. Nevertheless the proviral vector pIB120HIV may be unstable in recA+ *E. coli* host cells (Maschera, B., *et al.* J. Virol. (1995) *69*, 5431-5436. Therefore the stability of the pGEMT3-ΔPRT construct should be verified after every new preparation of plasmid.

HIV-HXB2 sequence:

The region of interest within the HIV-HXB2D sequence (nucleotide 1800 to 4400) is represented in Fig. 3 (schematically) and Fig. 4 (complete sequence). The location of several genes, restriction sites, primers and deletions (ΔPro, ΔRT, ΔProRT) are also indicated.

The sequence of HIV-1 (isolate HXB2, reference genome, 9718bp) was obtained from the National Center for Biotechnology Information

(NCBI), National Library of Medicine, National Institutes of Health *via* the ENTREZ Document Retrieval System.

Genbank name: HIVHXB2CG
Genbank Accesion No: Ø3455
NCBI Seq.ID No: 327742

Regions of recombination:

In combination with RT-PCR fragments generated by RVP5 and OUT3/IN3 primers, the pGEMT3-ΔPRT vector can be used to transfect MT4 cells as described in Example 1, Section 3. The region for recombination at the 5'-end of ΔProRT contains 188 nucleotides. The region for recombination at the 3'-end of ΔProRT is similar to the one described earlier (Kellam, P. and Larder, B.A. (1994) *supra*) and contains 130 nucleotides.

The length of these regions for recombination is not unimportant. Previous data (Bandyopadhyay, P.K. *et al.* Proc. Natl. Acad. Sci. U.S.A., (1984) *81*, 3476-3480; Rubnitz, J. and Subramani, S. (1984) *supra*) demonstrate that a 10-fold reduction in recombination frequency may occur when sequence homology is reduced from 214 to 163 base pairs. Furthermore, sufficient recombination events should occur within the electroporated cells to ensure that the generated viral phenotype is a reliable reflection of the quasi-species present in the treated HIV-positive patient. Optimisation of recombinant events can first be achieved by adjusting the ratio of linearised proviral vector to RT-PCR fragment that is used for electroporation of the target cells. The standard method therefore,with typical results of outcome, has previously been described by Kellam, P. and Larder, B.A. (1994 *supra*). As a consequence, it was decided to increase the initial input of about 2µg of PCR product (with 10µg of vector) to about 5µg or more. The result thereof was reflected in a faster appearance of visible virus growth (cytopathogenic effect) in the culture of transfected cells.

Another option for optimisation of recombination events would be the design of primers resulting in longer recombination sequences.

Nevertheless, the real input in the transfection reaction always depends on the yield after PCR. Some samples have a high yield and as a result there will be a higher input of amplified material in the transfection reaction (with better results on efficiency of recombination). However, despite a lower recombination efficiency, samples having a low yield can also be transfected and will result in viable virus with a reliable reflection of the virus population.

Example 3

Alternative primers for RT-PCR of the ProRT sequence:

New primers (A-D) have been designed relative to those used in Example 2 and should result in longer recombination sequences at both 5' and 3' end of the ProRT region. Two primers were designed at both the 5' and 3' end of the respective region to allow nested PCR. As indicated in Figs. 3 and 4 the direct repeat present at the 5' end of the ProRT region was taken into account when designing the respective primers. The new primers are as follows:

A	PRTO-5	: 5'-GCCCCTAGGA-AAAAGGGCTG-TTGG	(SEQ ID NO: 3)
B	PRTI-5	: 5'-TGAAAGATTG-TACTGAGAGA-CAGG	(SEQ ID NO: 4)
C	PRTI-3	: 5'-GATATTTCTC-ATGTTCATCT-TGGG	(SEQ ID NO: 5)
D	PRTO-3	: 5'-AGGTGGCAGG-TTAAAATCAC-TAGC	(SEQ ID NO: 6)

Example 4

Construction of an alternative ΔProRT vector:

As mentioned above, construction of an alternative ProRT deleted vector can be achieved by oligonucleotide-mediated mutagenesis. However, it is also possible to enlarge the ProRT deletion from the current 3'-end to the next KpnI site in the RT gene (40 base pairs further

downstream). Ligation of a Klenow-treated KpnI site to a Klenow-treated BstEII site will restore the initial BstEII recognition sequence. As such, this alternative vector behaves similarly to the pGMT3-ΔPRT vector described in Example 2, but has a slightly larger RT deletion.

Example 5

An HIV infected patient who received AZT from December 1989 until an undocumented later date, and switched to a combined chemotherapy of AZT + 3TC (1:1) from February 1994 until October 1995 donated plasma whose susceptibility to a number of RT inhibitors was determined according to the above described protocol of Example 1. Recombinant wild type HIV strain recIIIB was used in said protocol as a reference HIV virus. Table 1 shows the IC_{50} values (μM) measured and the ratio of said values. The Antivirogram is shown in Fig. 5.

Table 1

	Anti-HIV-1 activity IC_{50} (μM)				
Drug	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
loviride	0.1	0.12	0.11	0.05	2
tivirapine	0.019	0.018	0.019	0.01	1.5
AZT	0.001	0.002	0.002	0.004	0.4
3TC	31.6	100	65.8	0.56	118
d4T	0.07	0.49	0.06	0.12	0.5
ddI	2.0	0.8	1.4	2.83	0.5
ddC	0.2	0.2	0.2	0.38	0.5
AZT+3TC (1:1)	0.001	0.001	0.001	0.002	0.5

From these data, one can determine that monotherapy with 3TC is unlikely to benefit this particular patient. Combined therapy of AZT + 3TC (the current therapy), however, is still likely to exert a positive effect.

Example 6

A drug-naive HIV infected patient donated plasma whose susceptibility to a number of RT inhibitors was determined according to the above described protocol of Example 1. Recombinant wild type HIV strain recIIIB was used in said protocol as a reference HIV virus. Table 2 shows the IC_{50} values (μM) measured and the ratio of said values. An Antivirogram was prepared and shown in Fig. 6.

Table 2

Drug	Anti-HIV-1 activity IC_{50} (μM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
3TC	1.81	2.02	1.91	3.08	1
ddI	3.07	4.47	3.77	8.58	0.4
ddC	1.45	1.47	1.46	2.21	1
AZT	0.04	0.05	0.05	0.06	1
d4T	1.31	0.97	1.14	1.74	1
AZT+3TC (1:1)	0.05	0.04	0.05	0.02	3
DDC+D4T (1:1)	0.62	0.44	0.53	0.77	1
3TC+d4T (1:1)	0.42	0.44	0.43	1.11	0.4

From these data one can determine that the patient is infected with HIV strains closely resembling the wild type HIV. None of the drug regimens is to be excluded, so chemotherapy can be initiated with a drug such as AZT having a positive track record.

Example 7

A drug-naive HIV-infected patient donated plasma whose susceptibility to a number of RT inhibitors was determined according to the protocol set out in Example 1. Recombinant wild type HIV strain

recIIIB was used as a reference HIV virus. Table 3 shows the IC_{50} values (μM) measured and the ratio of said values. A bar graph showing relative change in drug susceptibility is shown in Fig. 7A. An Antivirogram was also prepared and is shown in Fig. 7B.

Table 3

	Anti-HIV-1 activity IC_{50} (μM)				
Drug	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
AZT	0.052	0.050	0.051	0.023	2
3TC	2.173	ND	2.173	1.381	2
ddI	0.475	0.429	0.452	0.648	0.7
ddC	1.042	1.389	1.216	1.616	0.8
d4T	1.142	1.657	1.399	1.368	1
loviride	0.035	0.025	0.030	0.024	1
tivirapine	0.042	0.049	0.046	0.021	2

From these data one can determine that the patient is infected with a HIV strain closely resembling the wild type HIV. None of the drug regimens is to be excluded, so chemotherapy can be initiated with a drug such as AZT, 3TC or others having a positive track record.

Example 8

An HIV-infected patient with a therapy history including AZT, 3TC and loviride donated plasma whose susceptibility to a number of RT inhibitors was determined according to the protocol set out in Example 1. Recombinant wild type HIV strain recIIIB was used as a reference HIV virus. Table 4 shows the IC_{50} values (μM) measured and the ratio of said values. A bar graph showing relative change in drug susceptability is shown in Fig. 8A. An Antivirogram was also prepared and is shown in Fig. 8B.

Table 4

Drug	Anti-HIV-1 activity IC_{50} (μM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
AZT	18.264	22.251	20.257	0.084	241
3TC	> 100.000	> 100.000	> 100.000	6.304	> 16
ddI	26.861	15.435	21.148	1.586	13
ddC	9.290	8.506	8.898	1.931	5
d4T	7.500	7.097	7.298	5.465	1
loviride	> 100.000	> 100.000	> 100.000	0.037	> 2717
tivirapine	1.626	1.604	1.615	0.021	78

From this data one can determine that the patient is infected with a HIV strain displaying a decreased susceptibility towards most of the nucleoside and non-nucleoside antiretroviral drugs examined. Therapy can still be initiated with D4T or DDC. The possibility of including protease-inhibitors into the therapy can be considered.

Example 9

An HIV-infected patient with a therapy history including multiple nucleoside analogue RT-inhibitors donated plasma whose susceptibility to a number of RT inhibitors was determined according to the protocol set out in Example 1. Recombinant wild type HIV strain recIIIB was used as a reference HIV virus. Table 5 shows the IC_{50} values (μM) measured and the ratio of said values. A bar graph showing relative change in drug susceptibility is shown in Fig. 9A. An Antivirogram was also prepared and is shown in Fig. 9B.

Table 5

Drug	Anti-HIV-1 activity IC_{50} (μM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
AZT	> 100.000	ND	> 100.000	0.291	> 344
3TC	> 100.000	> 100.000	> 100.000	16.670	> 6
ddI	> 100.000	> 100.000	> 100.000	4.757	> 21
ddC	60.079	73.049	66.564	3.444	19
d4T	> 100.000	> 100.000	> 100.000	12.030	> 8
loviride	0.064	0.058	0.061	0.065	0.9
tivirapine	0.052	0.043	0.048	0.042	1

From this data one can determine that the patient is infected with a HIV strain displaying a decreased susceptibility towards all nucleoside analogue antiretroviral drugs. Non-nucleoside antiretroviral drugs should not be excluded from therapy. Here also, the possibility of including protease inhibitors into the therapy can be considered.

Example 10

A drug-naive HIV-infected patient donated plasma whose susceptibility to a number of RT inhibitors and protease inhibitors was determined according to the protocol set out in Example 1. Recombinant wild type HIV strain recIIIB was used as a reference HIV virus. Table 6 shows the IC_{50} values (μM) measured and the ratio of said values. A bar graph showing relative change in drug susceptibility is shown in Fig. 10A. An Antivirogram was also prepared and is shown in Fig. 10B.

Table 6

Drug	Anti-HIV-1 activity IC_{50} (μM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio(1)/(2)
AZT	0.019	0.019	0.019	0.041	0.5
3TC	1.525	1.718	1.622	4.608	0.4
saquinavir	0.003	0.003	0.003	0.006	0.4
ritonavir	0.022	0.017	0.019	0.033	0.6
indinavir	0.013	0.013	0.013	0.016	0.8

From these data one can determine that the patient is infected with HIV strains closely resembling the wild type HIV. None of the drug regimens is to be excluded, so that chemotherapy can be initiated with a drug such as AZT, 3TC or others having a positive track record.

Example 11

An HIV infected patient with a therapy history including RT and protease inhibitors donated plasma whose susceptibility to a number of RT inhibitors and protease inhibitors was determined according to the protocol set out in Example 1. Recombinant wild type HIV strain recIIIb was used as a reference HIV virus. Table 7 shows the IC_{50} values (μM) measured and the ratio of said values. A bar graph showing relative change in drug susceptibility is shown in Fig. 11A. An Antivirogram was also prepared and is shown in Fig. 11B.

Table 7

Drug	Anti-HIV-1 activity IC_{50} (μM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
AZT	ND	0.015	0.015	0.047	0.3
3TC	> 100.000	93.962	96.981	5.178	19
saquinavir	0.014	0.015	0.014	0.012	1
ritonavir	1.198	1.739	1.468	0.062	24
indinavir	0.229	0.416	0.323	0.027	12

From these data one can determine that the patient is infected with a HIV strain displaying a decreased susceptibilty towards the RT-inhibitor 3TC and protease inhibitors indinavir and ritonavir. Accordingly, chemotherapy can be adjusted with drugs such as AZT or saquinavir having a positive track record.

Example 12

An HIV infected patient with a therapy history including RT and protease inhibitors donated plasma whose susceptibility to a number of RT inhibitors and protease inhibitors was determined according to the protocol set out in Example 1. Table 8 shows the IC_{50} values (μM) measured and the ratio of said values. A bar graph showing relative change in drug susceptibility is shown in Fig. 12A. An Antivirogram was also prepared and is shown in Fig. 12B.

Table 8

Drug	Anti-HIV-1 activity IC_{50} (μM)				
	Exp. 1	Exp. 2	Mean (1)	recIIIB ref (2)	ratio (1)/(2)
AZT	3.833	3.355	3.594	0.041	88
3TC	> 100.000	> 100.000	> 100.000	4.608	22
saquinavir	0.350	0.352	0.351	0.006	56
ritonavir	1.610	1.530	1.570	0.033	47
indinavir	0.124	ND	0.124	0.016	8

From these data one can determine that the patient is infected with a HIV strain displaying a decreased susceptibility towards RT-inhibitors 3TC and AZT and protease inhibitors indinavir, ritonavir and saquinavir.

Example 13

Comparison of Phenotyping relative to Genotyping

Plasma samples were obtained from HIV-infected individuals who had been receiving non-nucleoside RT inhibitor (NNRTI) long-term monotherapy. HIV-RNA was extracted, reverse-transcribed and amplified as described in Example 1. Starting from outer PCR material of positive samples, the first 785 nucleotides of the RT gene were amplified and this material was further used for genotyping.

Briefly, the 785 nucleotide fragment was subjected to cycle-sequencing reactions using the ThermoSequenase (ThermoSequenase is a Trade Mark) fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP from Amersham (cat# RPN2438). Four sequencing primers, chosen to allow for sequence determination in both directions from nucleotide 27 to nucleotide 681 of the RT gene, were used for each sample. The reactions were analysed on an ALF (ALF is a Trade Mark) automatic sequencer (Pharmacia). The generated sequences were exported to a Power Macintosh and further analysed with the

GeneWorks 2.5 software (Oxford Molecular Group Inc.). Resulting amino acid sequences were compared with the corresponding sequence of the laboratory HIV-1 clone HXB2D and resistance-associated mutations identified in patient material. The results are shown in Table 9 where the one-letter amino acid code is used.

Table 9

P	RESISTANCE ASSOCIATED MUTATIONS													FOLD RESISTANCE TO			
	M 41	D 67	K 70	A 98	K 101	K 103	V 108	E 138	Y 181	M 184	G 190	T 215	K 219	AZT	3TC	NNRTI 1	NNRTI 2
1						N								1	1	56	437
2						S								1	0.4	102	53
3						N								0.4	1	36	87
4														0.3	0.1	1	0.4
5														1	0.4	4	3
6						N								1	0.3	103	245
7						S								1	0.04	112	57
8						N								1	1	30	81
9									C					2	1	>1432	12
10						N								0.4	0.1	53	172
11	L	N	R			N				V		F	Q	94	>8	321	669
12	L											Y		28	2	1	3
13					E	K/N		A			G/A		Not Det	1	1	>1466	455
14						N								1	1	93	349
15						N								2	1	>2424	449
16						N				V				1	>8	21	115
17						N								1	1	29	102
18						S								1	1	95	181
19						N								1	1	78	260
20				G										0.4	1	22	25
21						N								1	0.4	47	68
22							I							1	0.4	3	3
23						N								0.2	0.2	9	9
24					Q									1	1	7	59

P = Patient

The top row of Table 9 shows the aminoacids (AA) found in the wild type sequence and their position. Amino acids changes at these positions are shown for each patient in the following rows. Only the positions at which changes were observed in patient material are shown. The right part of Table 9 presents the fold resistance to different RT inhibitors as determined by the method according to the invention for each of the patients' samples. NNRTI 1 is the non-nucleoside RT inhibitor that was administered to the patients. NNRTI 2 is another non-nucleoside RT inhibitor for which cross-resistance with the first one was observed to some extent.

The genotyping results regarding nucleoside analog RT inhbitors resistance are as follows :

- M41L, D67N, K70R, T215F/Y and K219Q/E are AZT resistance-associated mutations (Larder, B. and Kemp, S. (1989) Science *246*, 1155-1158; Kellam, P. *et al.* (1992) PNAS 89, 1934-1938). Their presence, individually or in different combinations, in the genome of HIV isolated from patient material correlates with the phenotypic resistance as determined by the Antivirogram generated (patients 11 and 12).

- The same applies to resistance to 3TC associated with the M184V mutation (Tisdale, M. *et al.* (1993) PNAS *90*, 5653-5656) which is observed only in the patients which show phenotypic resistance to the drug (patients 11 and 16).

The genotyping results regarding NNRTIs resistance are as follows :

- Three patients (3, 4 and 12) have no NNRTI resistance-associated mutation and are phenotypically sensitive to the drug.

- Most of the patients who show phenotypic resistance to the NNRTIs have a NNRTI resistance-associated mutation at position 103 (K103N/S).

- One patient (9) has the Y181C NNRTI resistance-associated mutation and shows a high phenotypic resistance (>1432 fold) to NNRTI 1.

- Patient 13 has several NNRTI resistance-associated mutations (K101E, K103N partially and G190A partially). This patient also shows a high phenotypic resistance (>1466 fold) to NNRTI 1. The E138A mutation observed in this sample is not associated so far with resistance. However, another mutation at this same position, i.e. E138K, has been demonstrated to play an important role in resistance to the TSAO compounds (Balzarini, J. *et al.* (1993) PNAS *90*, 6952-9656). The role of the E138A mutation still needs to be assessed.

- Patient 20 has the A98G NNRTI resistance-associated mutation and shows phenotypic resistance to the tested NNRTIs.

- Patient 22 has the V108I NNRTI resistance-associated mutation but does not show any phenotypic resistance to the tested NNRTIs.

- Patient 24 shows no NNRTI resistance-associated mutation (the K101Q mutation is found in several HIV-1 wild type genomes) but is phenotypically resistant to the tested NNRTIs.

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(ii) TITLE OF INVENTION: Method of managing the chemotherapy of patients who are HIV positive based on the phenotypic drug sensitivity of human HIV strains

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: EP 96200175.6
(B) FILING DATE: 26-JAN-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CATTGCTCTC CAATTACTGT GATATTTCTC ATG 33

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGGAAGATCT GGCCTTCCTA CAAGGG 26

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCCCCTAGGA AAAAGGGCTG TTGG 24

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TGAAAGATTG TACTGAGAGA CAGG 24

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GATATTTCTC ATGTTCATCT TGGG 24

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Human immunodeficiency virus type 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AGGTGGCAGG TTAAAATCAC TAGC 24

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMBP-COLLECTION
Page 1 of Form BCCM/LMBP/BP/4/96-07 Receipt in the case of an original deposit

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the International Depositary Authority BCCM/LMBP identified at the bottom of next page

International Form BCCM/LMBP/BP/4/96-07

To Name of the depositor : VIRCO nv.

Address : Drie Eikenstraat, 661
2650 Edegem

I. Identification of the microorganism:

I.1 Identification reference given by the depositor:

pGEMT3ΔPRT

I.2 Accession number given by the International Depositary Authority:

LMBP3590

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM LMBP-COLLECTION
Page 2 of Form BCCM/LMBP/BP/4/96-07 Receipt in the case of an original deposit

II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by:

(mark with a cross the applicable box(es)):

☒ a scientific description

☐ a proposed taxonomic designation

III. Receipt and acceptance

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) November 08, 1996

IV International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM)
Laboratorium voor Moleculaire Biologie - Plasmidencollectie (LMBP)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s)

Date : November 19, 1996

Lic Martine Vanhoucke

Claims: -

1. A method of managing HIV chemotherapy of patients who are HIV positive, which comprises transfecting a cell line susceptible to infection by HIV with a sequence from the pol gene of HIV obtained from a patient and a HIV-DNA construct from which said sequence has been deleted, culturing said transfected cells so as to create a stock of chimeric viruses, assessing the phenotypic sensitivity of said chimeric viruses to an inhibitor of said enzyme encoded by the pol gene of HIV and assigning a value thereto, constructing a data set comprising said value for chimeric virus sensitivity and the corresponding value for a chimeric wild-type strain of HIV, repeating the sensitivity assessment for at least two further inhibitors and thereby constructing at least three such data sets in total, representing said data sets in two dimensional or three dimensional graphical form such that the difference between the chimeric and wild-type sensitivities in the case of each data set provides a visual measure of the resistance of the chimeric stock to treatment by the inhibitor in question, and selecting the optimum inhibitor(s) on the basis of the graphical representation of the resistances so measured.

2. A method of managing HIV chemotherapy according to Claim 1, wherein the data sets are represented on a polygonal or quasi-circular graph comprising:

(a) a plurality of normalised axes extending radially from an origin, each axis corresponding to one data set or inhibitor or combination thereof;

(b) the axes being normalised such that the sensitivity values for wild-type HIV for the various inhibitors are equal on each axis, the data points for wild-type HIV being optionally represented and connected to form a regular polygon whose vertices lie on the axes and whose center is defined by the origin;

(c) on each axis a data point representing the sensitivity value of the chimeric HIV stock against the inhibitor corresponding to said axis is plotted, the chimeric data points being optionally connected to form a regular or irregular polygon the shape of which represents the resistance of the chimeric stock to a range of inhibitors.

3. A method according to Claim 2, wherein each axis has a logarithmic scale.

4. A method according to Claim 2 or 3, wherein eccentric data points in the chimeric polygon, if represented, identify inhibitors whose usefulness can be assumed to be of little or no benefit to the patient, while data points lying within, on or close outside the wild-type polygon identify inhibitors whose usefulness can be assumed to be of substantial benefit to the patient.

5. A method according to any preceding claim, wherein each of said three or more data sets further comprises a value for worst-case measurable resistance for the inhibitor in question, said worst case values being represented on said graphical representations such that the data point for the chimeric stock can be compared both to wild-type and to worst-case HIV, thereby providing an assessment of the relative value of the inhibitor in a particular case.

6. A method of managing HIV chemotherapy of patients who are HIV positive, which comprises the steps of:

(a) periodically assessing the phenotypic sensitivity of a patient's HIV strains according to any one of Claims 1-5;

(b) maintaining the chemotherapy with the selected inhibitor while the patient's HIV strains remain susceptible to the selected chemotherapy;

(c) selecting a different inhibitor if and when the susceptibility of the original inhibitor decreases.

7. A clinical management device for use in the management of chemotherapy of patients who are HIV positive, said device bearing a graphical representation of a plurality of data sets as defined in Claim 1.

8. A method according to any one of Claims 1-6, wherein the phenotypic sensitivity of said chimeric viruses to inhibitors of at least two enzymes encoded by the pol gene of HIV is simultaneously assessed.

9. A method of determining the phenotypic drug sensitivity of individual HIV strains in a patient to inhibitors of at least two enzymes encoded by the pol gene of HIV, which comprises transfecting a cell line susceptible to infection by HIV with a sequence from the pol gene of HIV obtained from a patient and a HIV-DNA construct from which said sequence has been deleted, culturing said transfected cells so as to create a stock of chimeric viruses and assessing the phenotypic sensitivity of said chimeric viruses to inhibitors of said enzymes encoded by the pol gene of HIV.

10. A method according to any one of Claims 1-6, 8 and 9, wherein said sequence from the pol gene is isolated from a sample of a biological material obtained from the patient whose phenotypic drug sensitivity is being determined.

11. A method according to Claim 10, wherein said biological material is selected from plasma, serum or a cell-free body fluid selected from semen and vaginal fluid.

12. A method according to Claim 10, wherein the biological material is whole blood to which an RNA stabiliser has been added.

13. A method according to Claim 10, wherein the biological material is tissue material selected from brain tissue or lymph nodal tissue.

14. A method according to any one of Claims 9-13, wherein the at least two enzymes are selected from HIV RT, protease and integrase.

15. A method according to any one of Claims 1-6 and 8-14, wherein the cell line susceptible to infection by HIV is a $CD4^+$ T-cell line.

16. A method according to Claim 15, wherein the $CD4^+$ T-cell line is the MT4 cell line or the HeLa $CD4^+$ cell line.

17. A method according to any one of Claims 1-6 and 8-16, wherein the desired region of the patient's pol gene is reverse transcribed using a specific downstream primer.

18. A method according to Claim 17, wherein the sequence to be reverse transcribed is that coding for reverse transcriptase and protease.

19. A method according to Claim 18, wherein the downstream primer is OUT3 : 5'-CAT TGC TCT CCA ATT ACT GTG ATA TTT CTC ATG-3' (SEQ ID NO: 1).

20. A method according to any one of Claims 17-19, wherein the product of reverse transcription is amplified using a nested PCR technique.

21. A method according to any one of Claims 1-6 and 8-20, wherein the HIV-DNA construct is one from which the RT and protease genes are deleted and is the plasmid pGEMT3-ΔPRT as deposited at the Belgian Coordinated Collections of Microorganisms-BCCM LMBP-Collection on November 8, 1996 under the number LMBP3590.

22. A method according to any one of Claims 1-6 and 8-21, wherein the transfection is achieved by electroporation.

23. A method according to any one of Claims 1-6 and 8-21, wherein the transfection is achieved by the use of cationic lipids.

24. A method according to any one of Claims 1-6 and 8-23, wherein the phenotypic drug sensitivity of the chimeric viruses to different RT, protease and integrase inhibitors is assessed in an automated cellular-based assay.

25. A method according to any one of Claims 1-6 and 8-24, wherein the phenotypic drug sensitivity of the chimeric viruses and of the wild HIV strain to one or more RT, protease or integrase inhibitor(s) is expressed as an inhibitory concentration (IC value).

26. A method according to any one of Claims 1-6 and 8-25, wherein RT inhibitors are selected from nucleoside RT inhibitors such as AZT, ddI, ddC, 3TC, d4T, non-nucleoside RT inhibitors such as loviride, nevirapine and tivirapine, protease inhibitors such as saquinavir, indinavir and ritonavir and integrase inhibitors such as caffeic acid phenylethyl ester (CAPE).

27. A method of managing HIV chemotherapy of patients who are HIV positive, substantially as hereinbefore described and exemplified.

28. A clinical management device, substantially as hereinbefore described and exemplified with particular reference to and as illustrated in Figs. 5-12 of the accompanying drawings.

29. A method of determining the phenotypic drug sensitivity of individual HIV strains in a patient to inhibitors of at least two enzymes encoded by the pol gene of HIV, substantially as hereinbefore described and exemplified.

Fig. 1

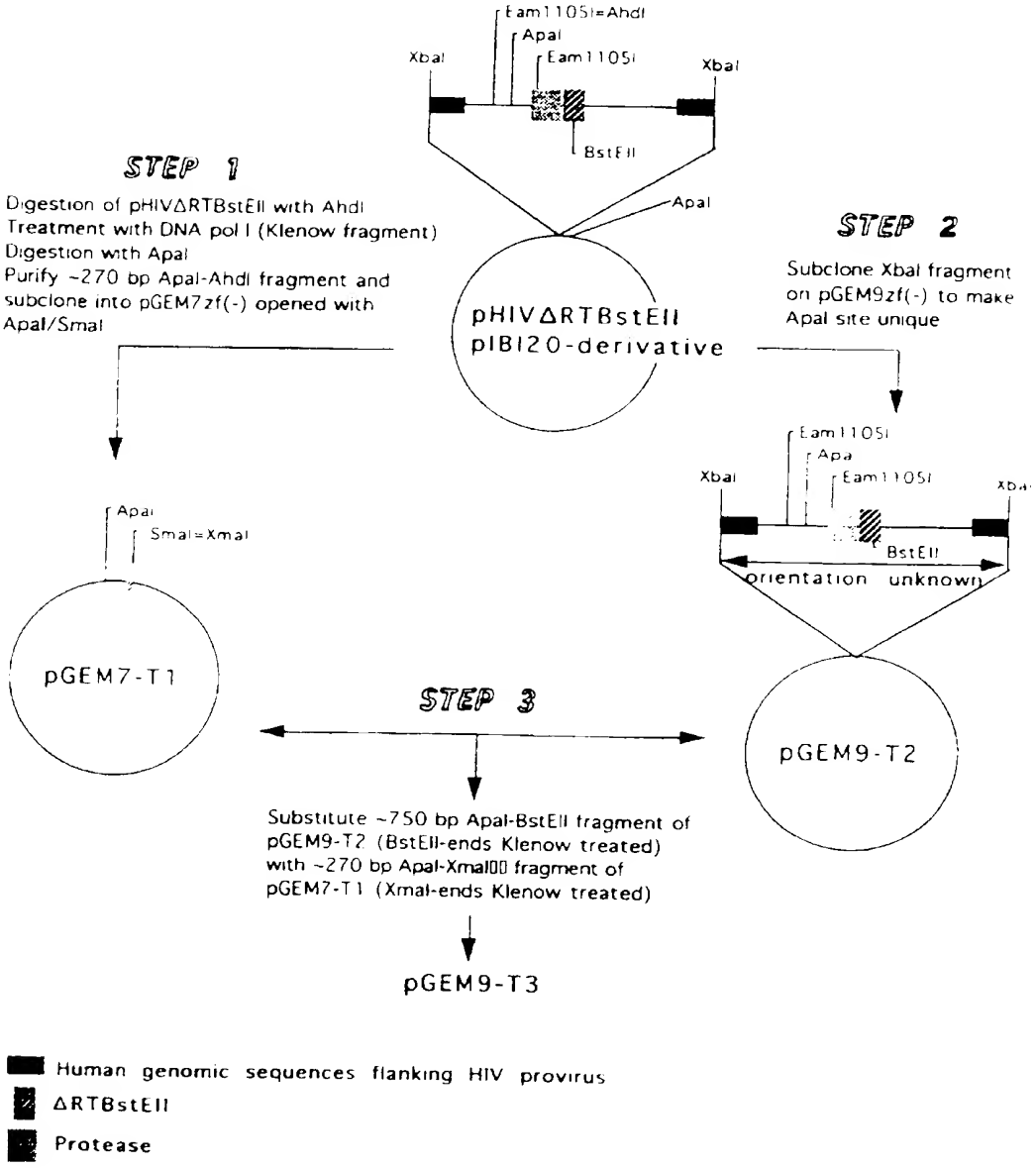

Eam1105I=AhdI
ApaI
Eam1105I
XbaI
XbaI
BstEII
ApaI
pHIVΔRTBstEII
pIBI20-derivative
STEP 1
Digestion of pHIVΔRTBstEII with AhdI
Treatment with DNA pol I (Klenow fragment)
Digestion with ApaI
Purify ~270 bp ApaI-AhdI fragment and subclone into pGEM7zf(-) opened with ApaI/SmaI
STEP 2
Subclone XbaI fragment on pGEM9zf(-) to make ApaI site unique
ApaI
SmaI=XmaI
pGEM7-T1
Eam1105I
ApaI
Eam1105I
XbaI
BstEII
orientation unknown
pGEM9-T2
STEP 3
Substitute ~750 bp ApaI-BstEII fragment of pGEM9-T2 (BstEII-ends Klenow treated) with ~270 bp ApaI-XmaI00 fragment of pGEM7-T1 (XmaI-ends Klenow treated)
pGEM9-T3
Human genomic sequences flanking HIV provirus
ΔRTBstEII
Protease

Fig. 2

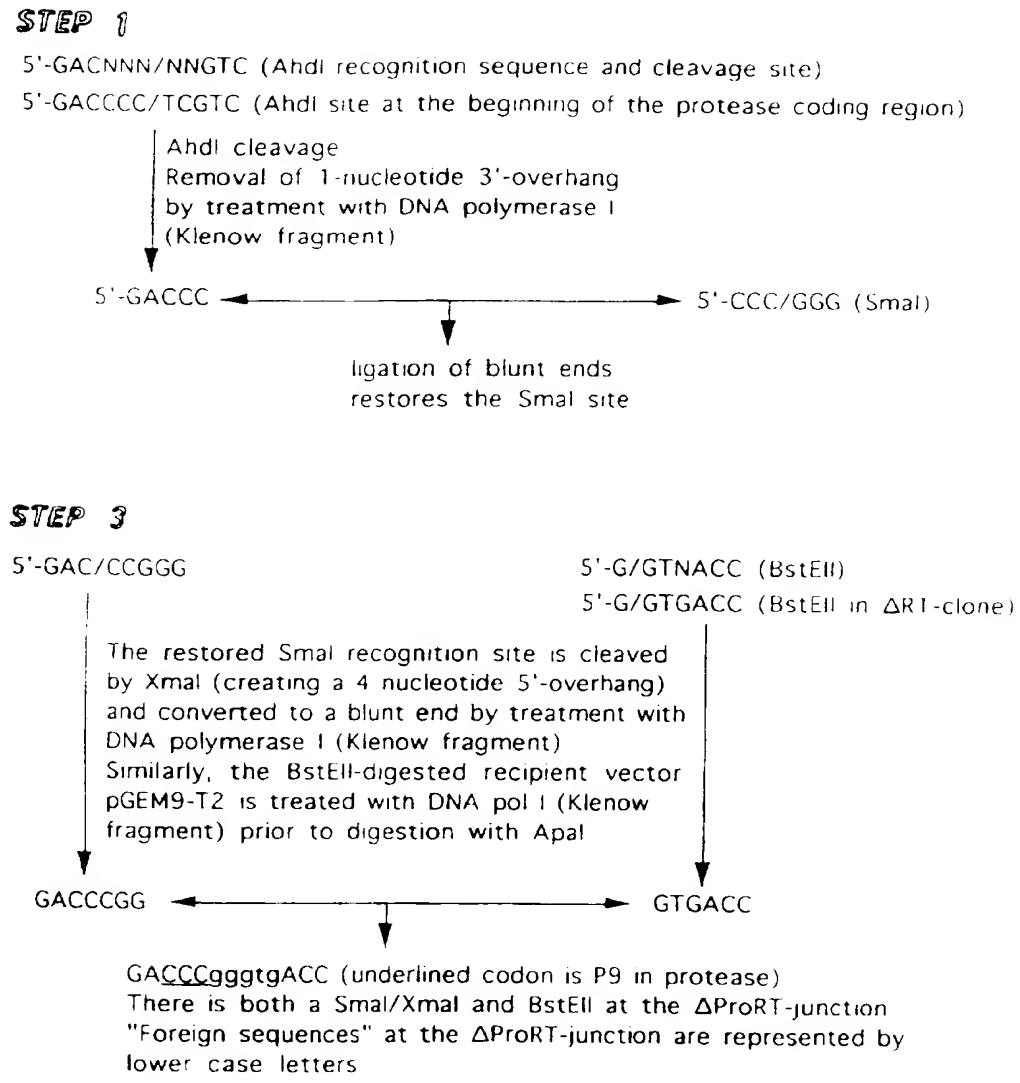
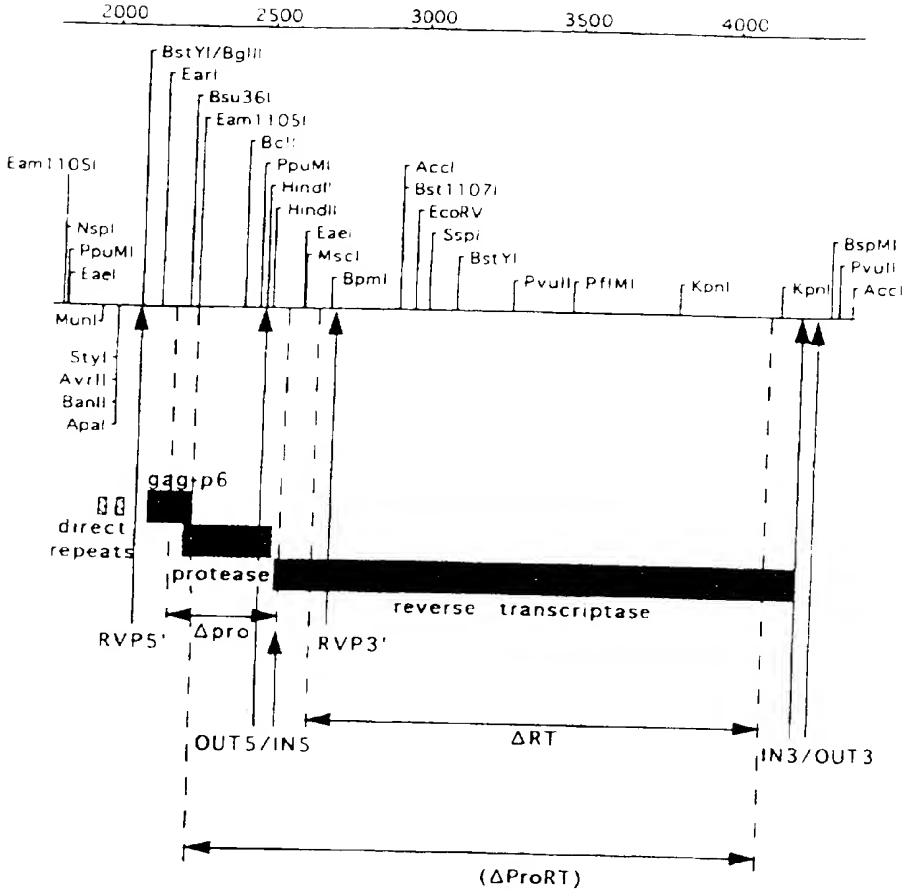

STEP 1
5'-GACNNN/NNGTC (AhdI recognition sequence and cleavage site)
5'-GACCCC/TCGTC (AhdI site at the beginning of the protease coding region)
AhdI cleavage
Removal of 1-nucleotide 3'-overhang
by treatment with DNA polymerase I
(Klenow fragment)
5'-GACCC
5'-CCC/GGG (SmaI)
ligation of blunt ends
restores the SmaI site
STEP 3
5'-GAC/CCGGG
5'-G/GTNACC (BstEII)
5'-G/GTGACC (BstEII in ΔRT-clone)
The restored SmaI recognition site is cleaved
by XmaI (creating a 4 nucleotide 5'-overhang)
and converted to a blunt end by treatment with
DNA polymerase I (Klenow fragment)
Similarly, the BstEII-digested recipient vector
pGEM9-T2 is treated with DNA pol I (Klenow
fragment) prior to digestion with ApaI
GACCCGG
GTGACC
GACCCgggtgACC (underlined codon is P9 in protease)
There is both a SmaI/XmaI and BstEII at the ΔProRT-junction
"Foreign sequences" at the ΔProRT-junction are represented by
lower case letters

Fig. 3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2500
3000
3500
4000
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EarI
Bsu36I
Eam1105I
BclI
PpuMI
HindI'
HindII
EaeI
MscI
BpmI
AccI
Bst1107I
EcoRV
SspI
BstYI
PvuII
PflMI
KpnI
KpnI
BspMI
PvuII
AccI
Eam1105I
NspI
PpuMI
EaeI
MunI
StyI
AvrII
BanII
ApaI
gag-p6
direct repeats
protease
reverse transcriptase
RVP5'
Δpro
RVP3'
OUT5/IN5
ΔRT
IN3/OUT3
(ΔProRT)

HXB2 (sequence range: 1800 to 4400)

Fig. 4A

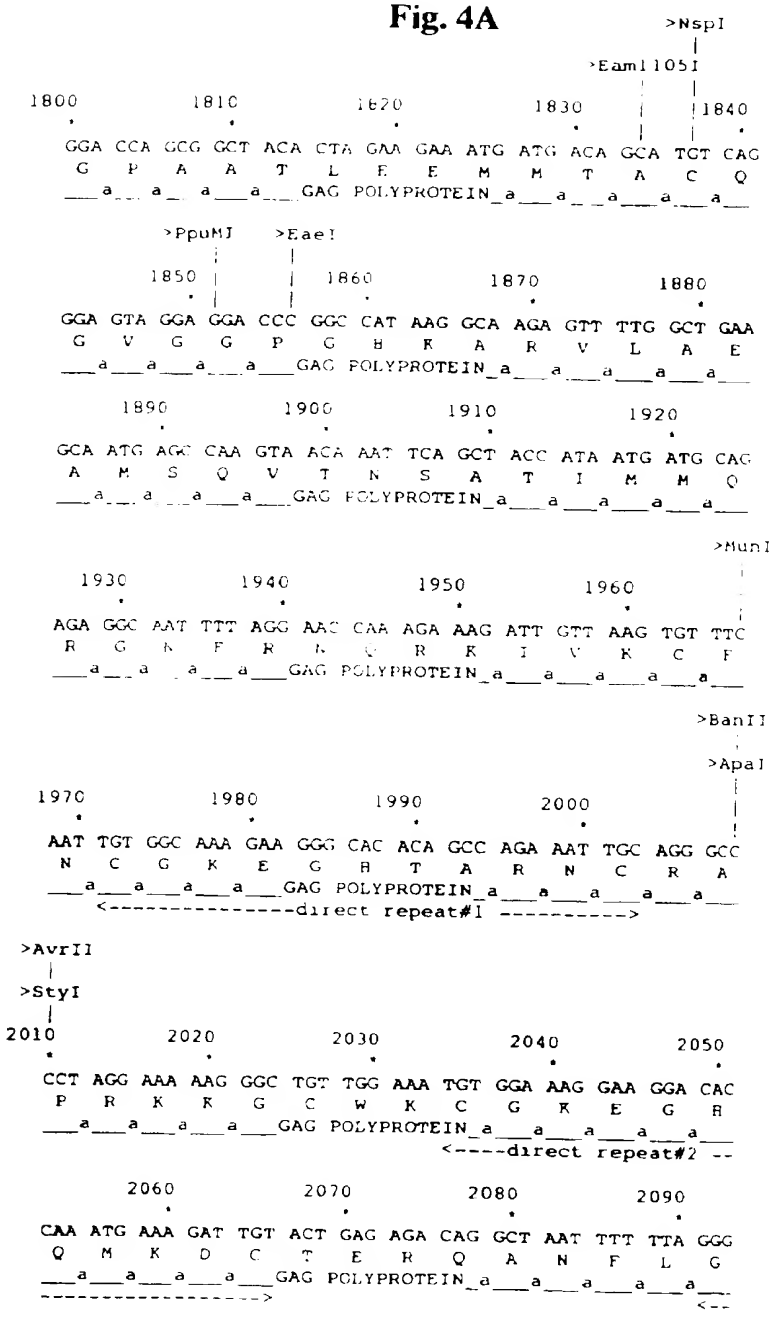

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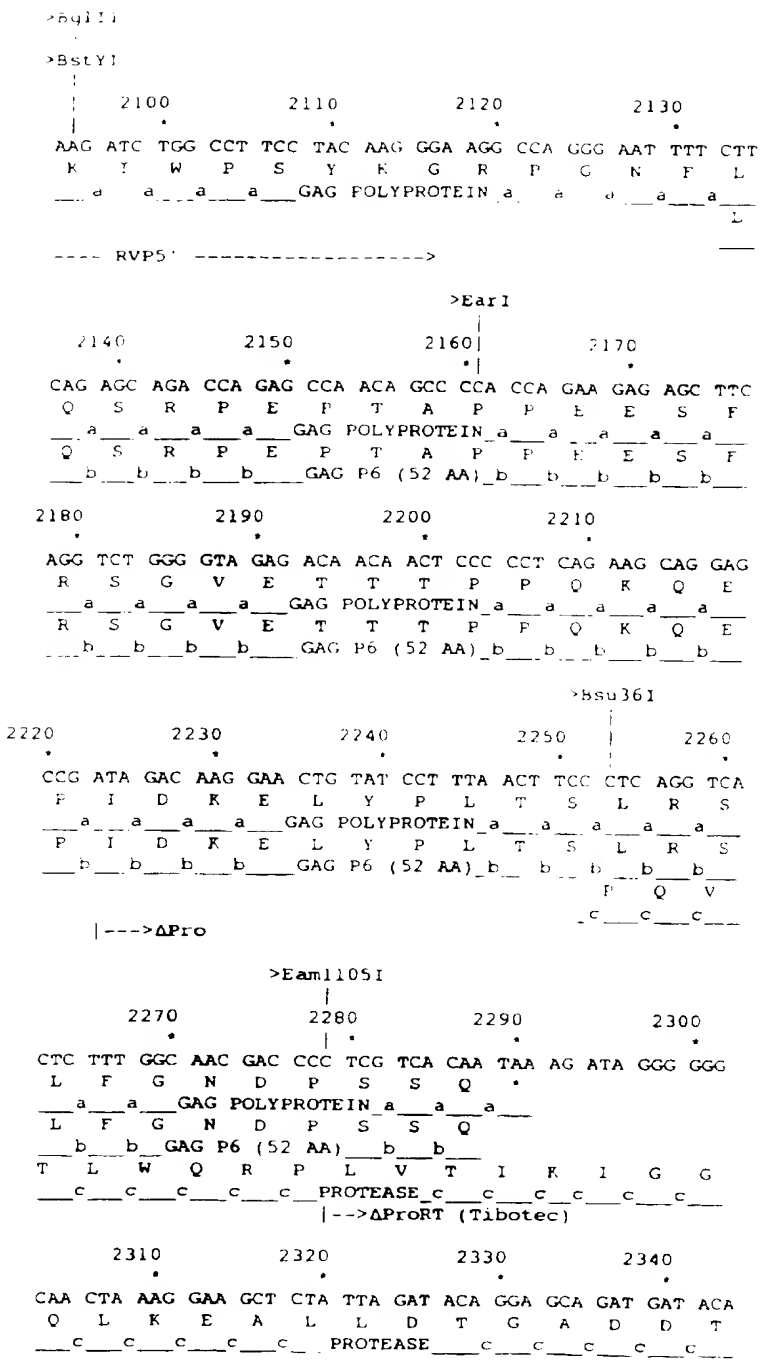
Fig. 4B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---- RVP5' ---------->
>EarI
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2150
2160
2170
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GAG P6 (52 AA)
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2190
2200
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GAG P6 (52 AA)
P Q V
|--->ΔPro
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2280
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2300
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L F G N D P S S Q
GAG POLYPROTEIN
GAG P6 (52 AA)
T L W Q R P L V T I K I G G
PROTEASE
|-->ΔProRT (Tibotec)
2310
2320
2330
2340
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PROTEASE

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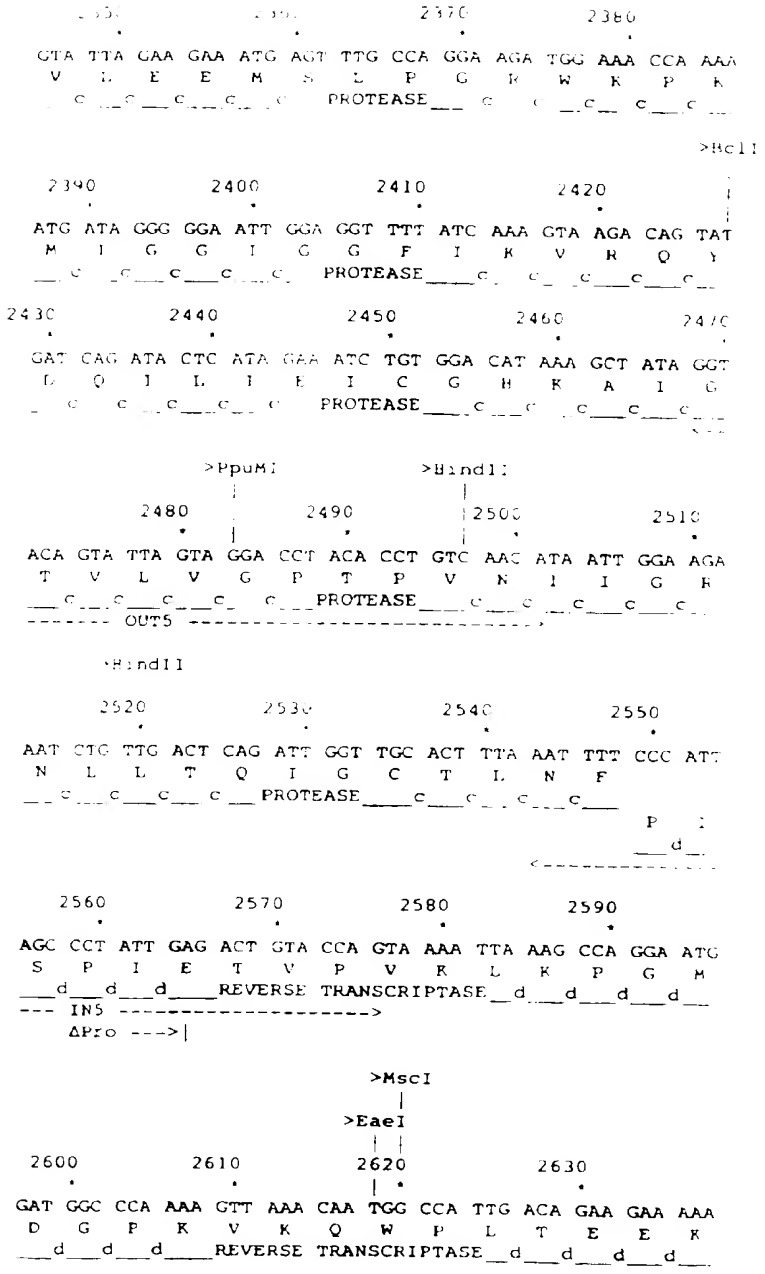
Fig. 4C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2400
2410
2420
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2440
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2460
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PROTEASE
>PpuMI
>HindII
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>EaeI
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Fig. 4D

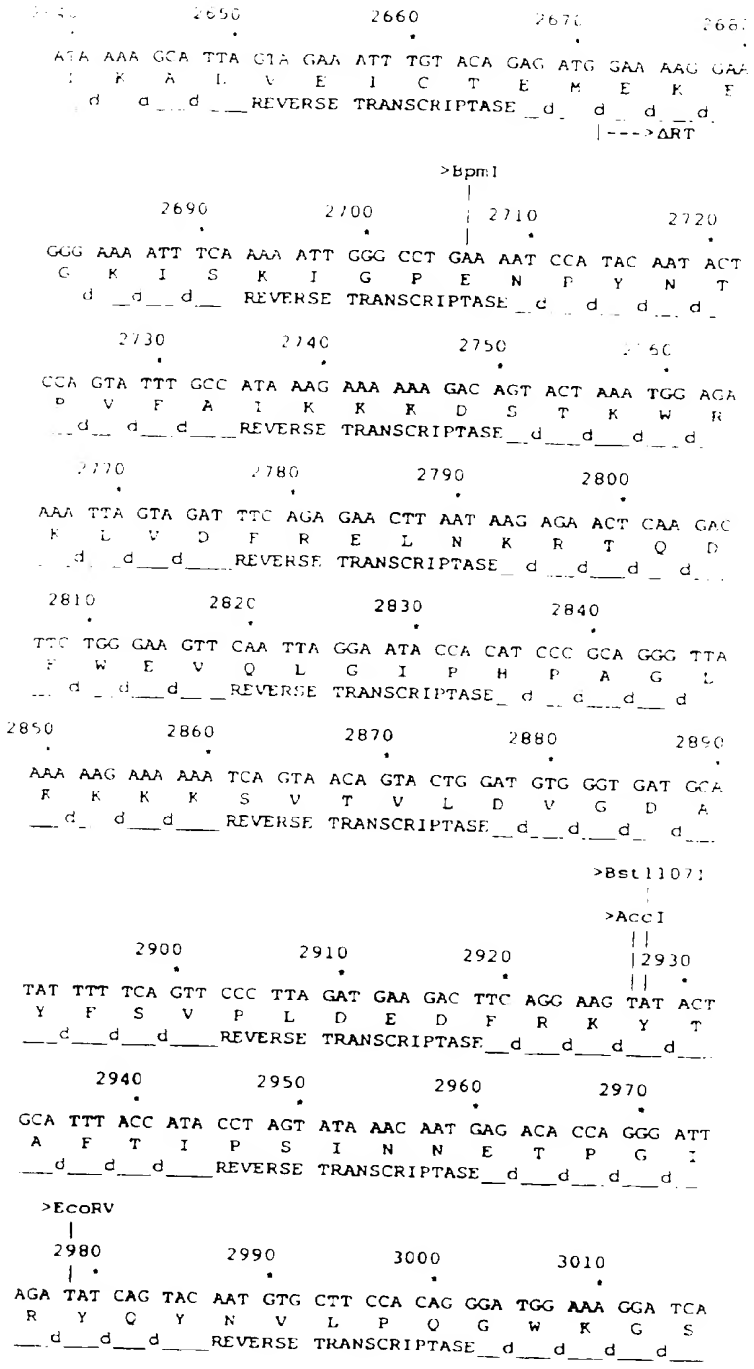
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Fig. 4E

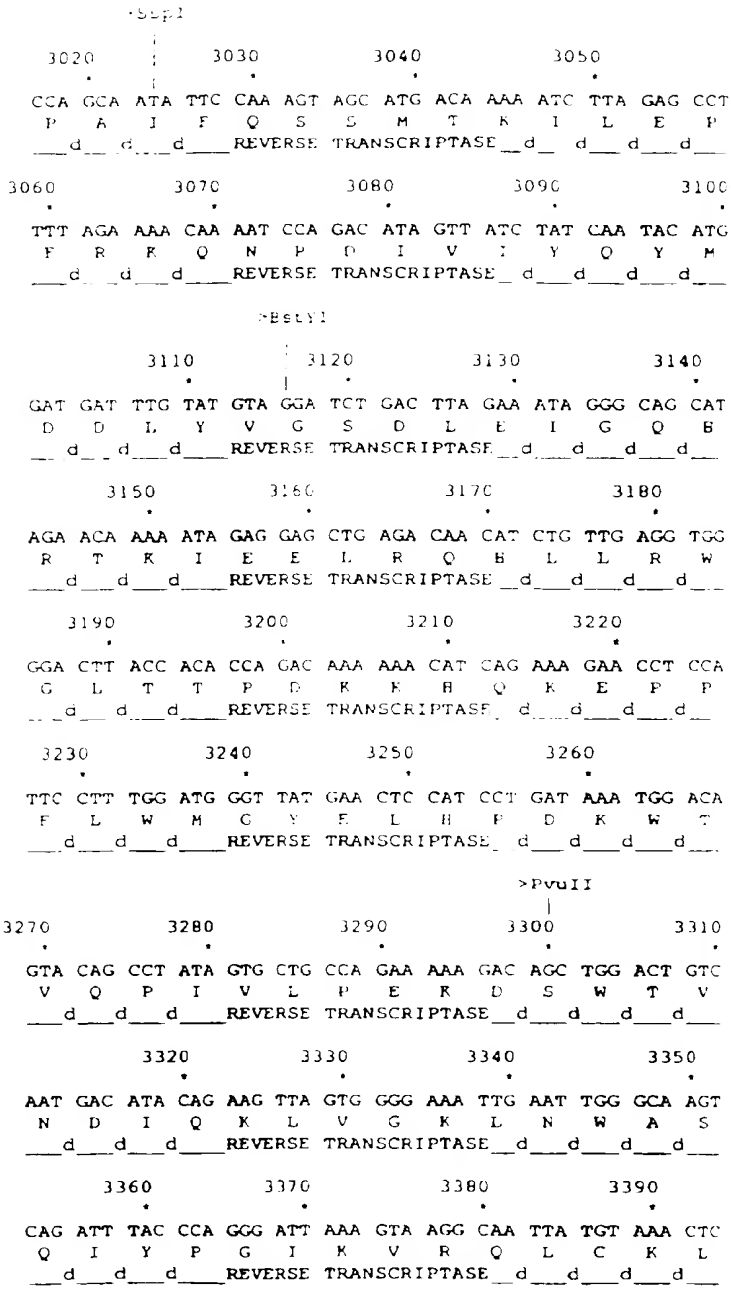
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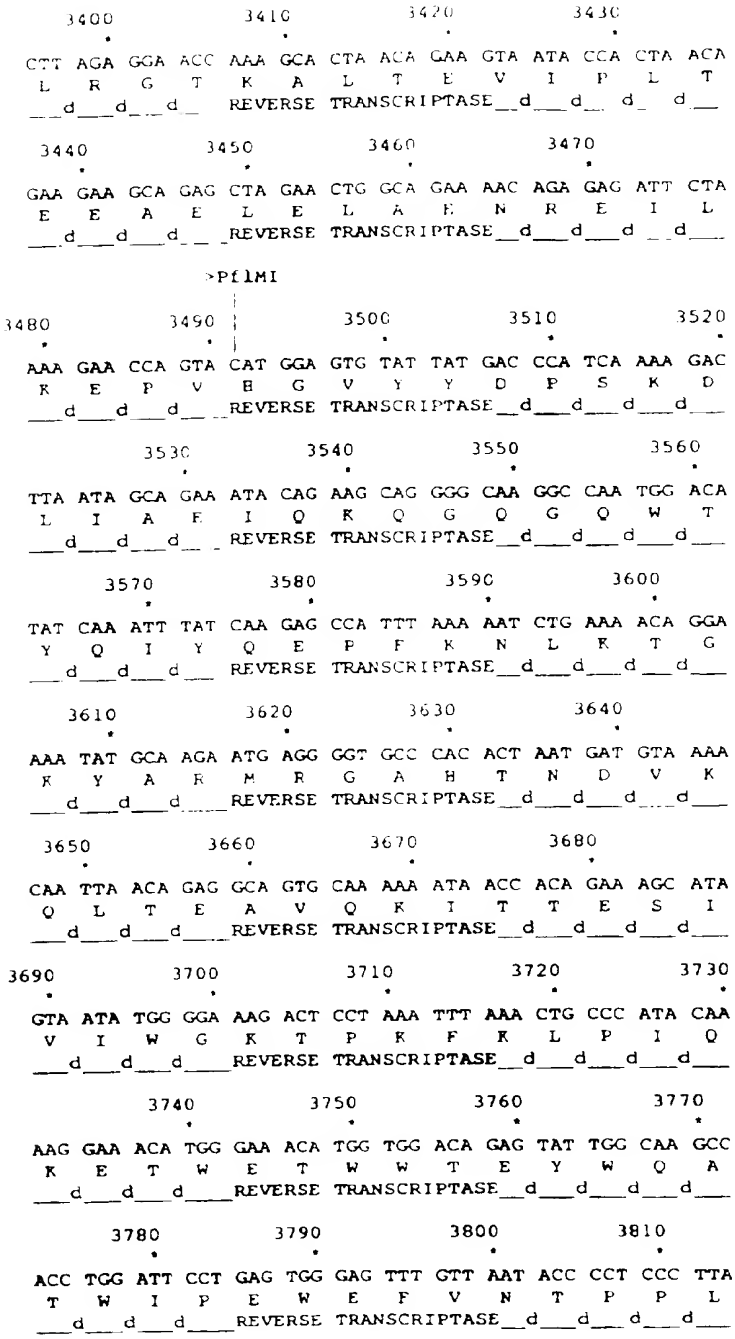
Fig. 4F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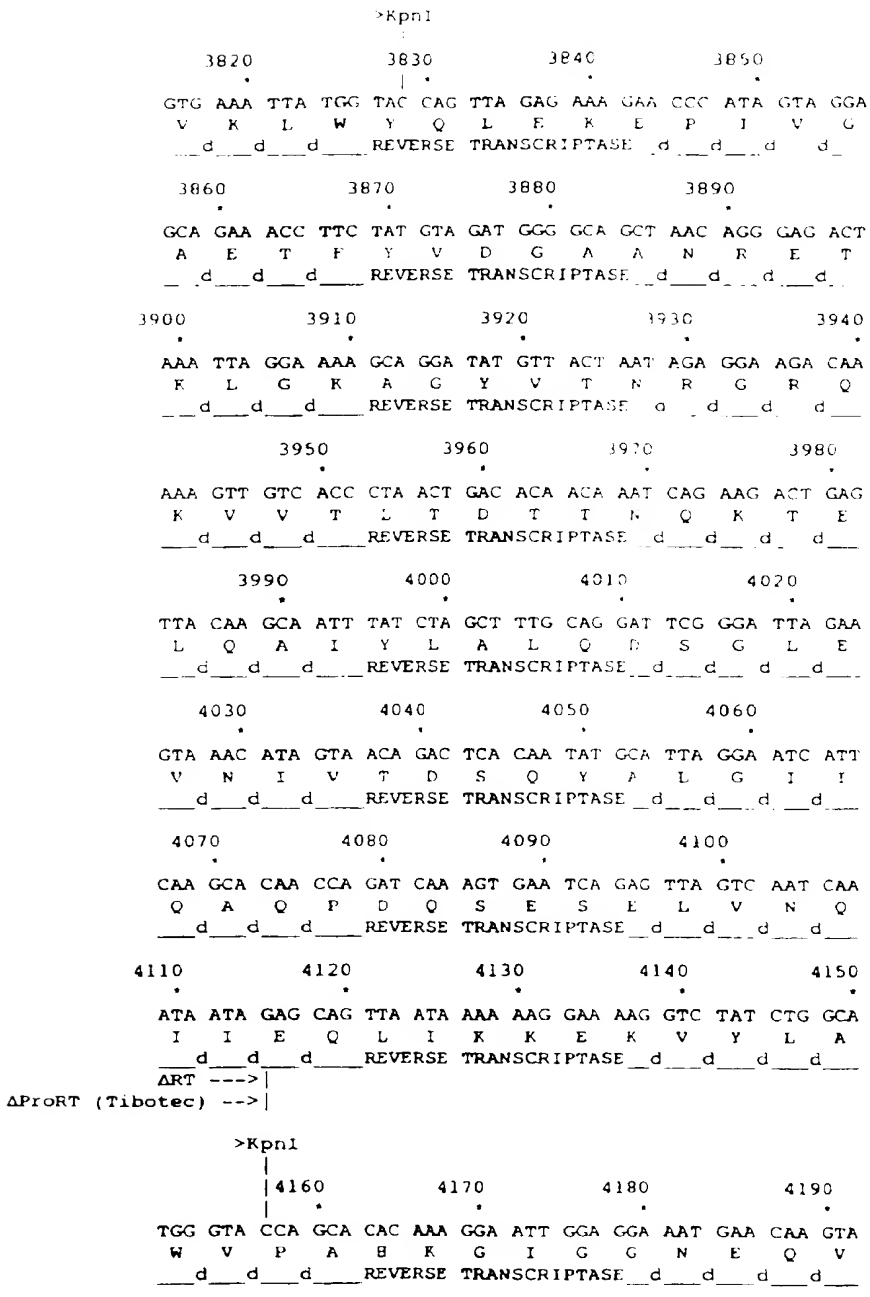
10/18

Fig. 4G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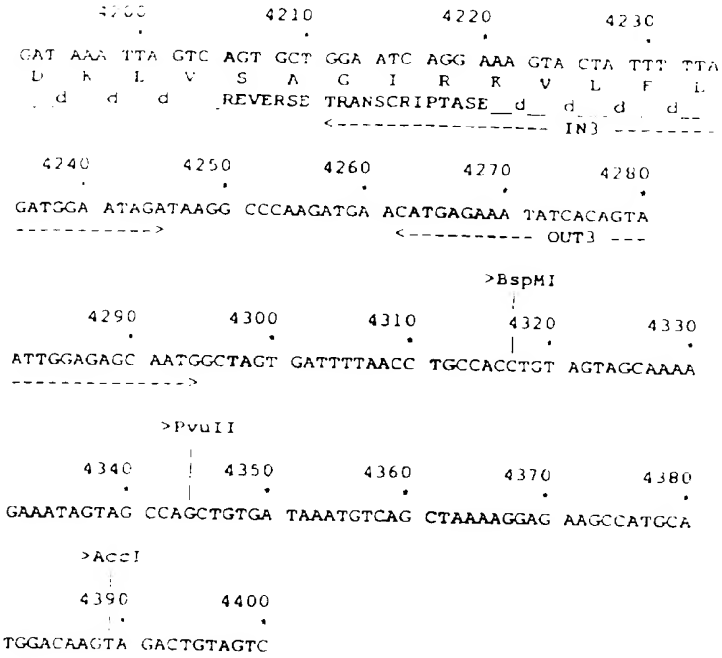
Q A Q P D Q S E S E L V N Q
REVERSE TRANSCRIPTASE
4110 4120 4130 4140 4150
ATA ATA GAG CAG TTA ATA AAA AAG GAA AAG GTC TAT CTG GCA
I I E Q L I K K E K V Y L A
REVERSE TRANSCRIPTASE
ΔRT --->|
ΔProRT (Tibotec) --->|
>KpnI
4160 4170 4180 4190
TGG GTA CCA GCA CAC AAA GGA ATT GGA GGA AAT GAA CAA GTA
W V P A H K G I G G N E Q V
REVERSE TRANSCRIPTASE

11/18

Fig. 4H


4200
4210
4220
4230
GAT AAA TTA GTC AGT GCT GGA ATC AGG AAA GTA CTA TTT TTA
D K L V S A G I R R V L F L
d d d REVERSE TRANSCRIPTASE d d d d
<---------- IN3 ---------
4240
4250
4260
4270
4280
GATGGA ATAGATAAGG CCCAAGATGA ACATGAGAAA TATCACAGTA
---------->
<---------- OUT3 ---
>BspMI
4290
4300
4310
4320
4330
ATTGGAGAGC AATGGCTAGT GATTTTAACC TGCCACCTGT AGTAGCAAAA
---------->
>PvuII
4340
4350
4360
4370
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GAAATAGTAG CCAGCTGTGA TAAATGTCAG CTAAAAGGAG AAGCCATGCA
>AccI
4390
4400
TGGACAAGTA GACTGTAGTC

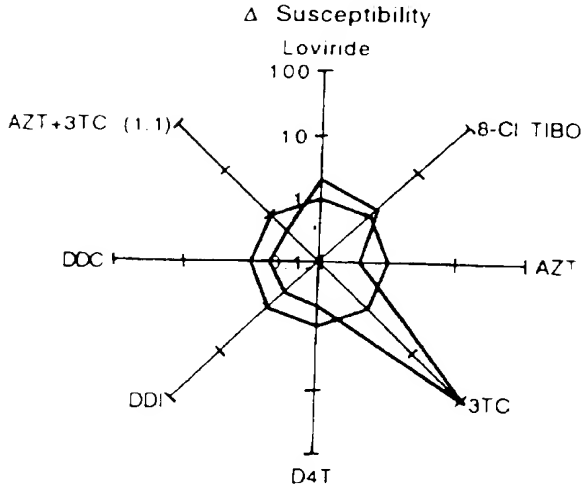
Δ Susceptibility
Loviride
100
10
1
0.1
0
AZT+3TC (1:1)
8-Cl TIBO
DDC
AZT
DDI
3TC
D4T

Fig. 5

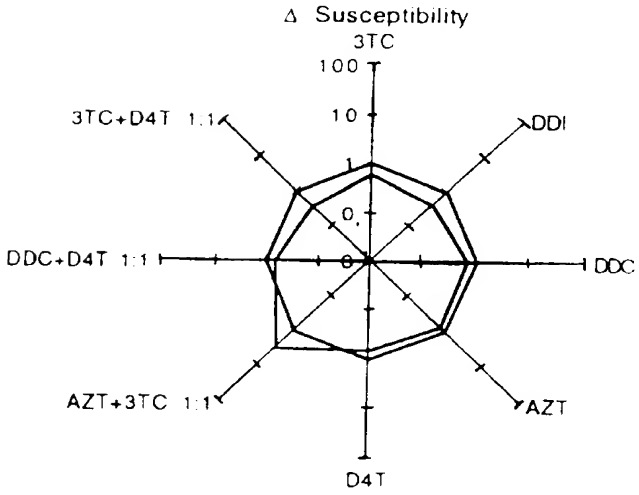
Δ Susceptibility
3TC
100
10
1
0.1
0
3TC+D4T 1:1
DDI
DDC+D4T 1:1
DDC
AZT+3TC 1:1
AZT
D4T

Fig. 6

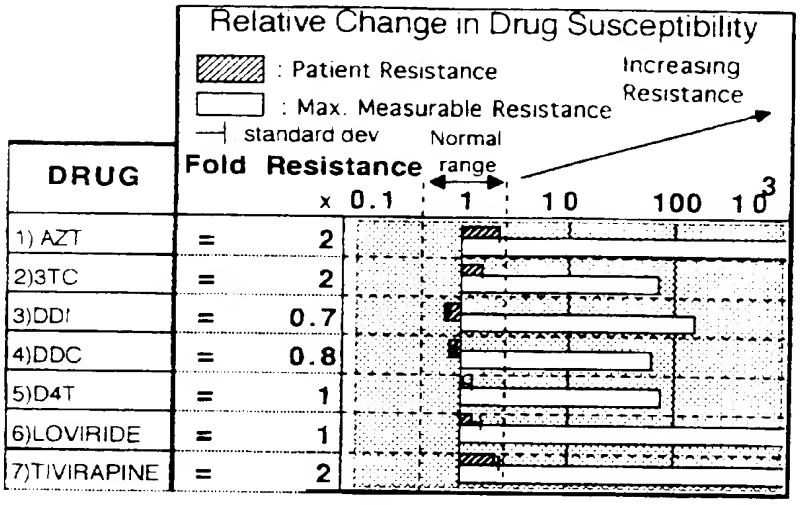
Relative Change in Drug Susceptibility
: Patient Resistance
: Max. Measurable Resistance
standard dev
Increasing Resistance
Normal range
DRUG
Fold Resistance
x 0.1
1
10
100
10 3
1) AZT = 2
2)3TC = 2
3)DDI = 0.7
4)DDC = 0.8
5)D4T = 1
6)LOVIRIDE = 1
7)TIVIRAPINE = 2

Fig. 7A

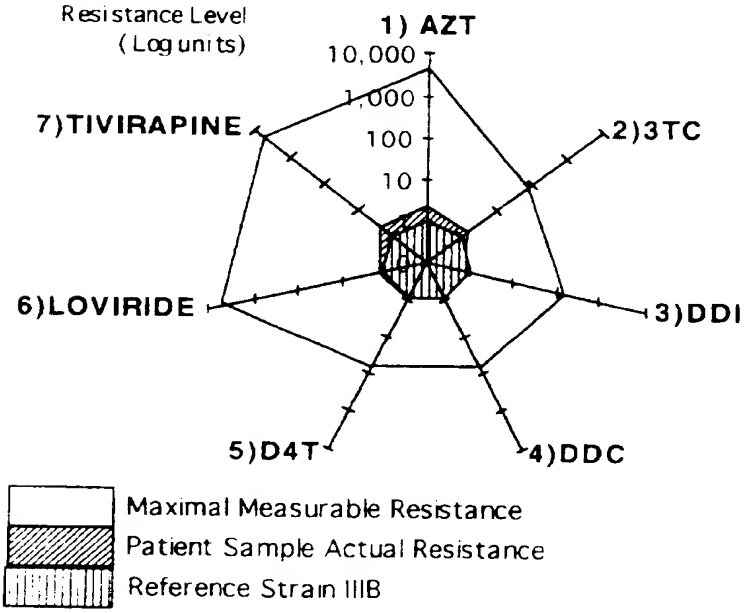
Resistance Level
(Log units)
1) AZT
10,000
1,000
100
10
2)3TC
3)DDI
4)DDC
5)D4T
6)LOVIRIDE
7)TIVIRAPINE
Maximal Measurable Resistance
Patient Sample Actual Resistance
Reference Strain IIIB

Fig. 7B

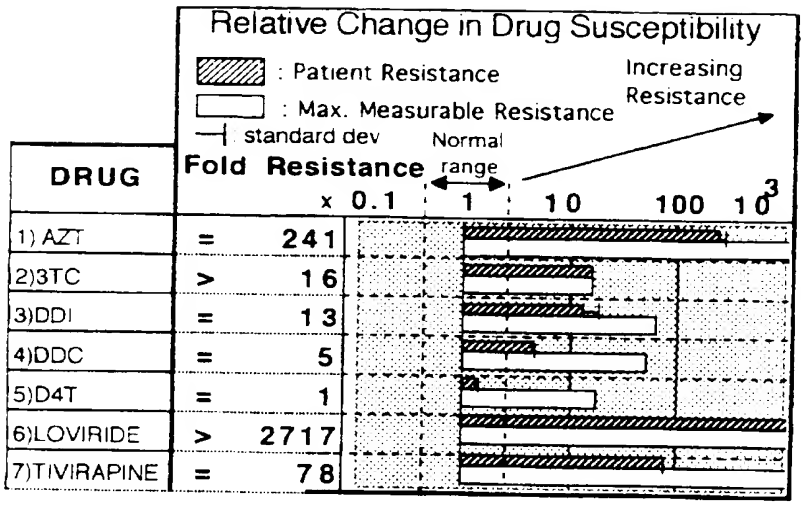
Relative Change in Drug Susceptibility
: Patient Resistance
: Max. Measurable Resistance
standard dev
Increasing Resistance
Normal range
Fold Resistance
x 0.1
1
10
100
10^3
DRUG
1) AZT = 241
2)3TC > 16
3)DDI = 13
4)DDC = 5
5)D4T = 1
6)LOVIRIDE > 2717
7)TIVIRAPINE = 78

Fig. 8A

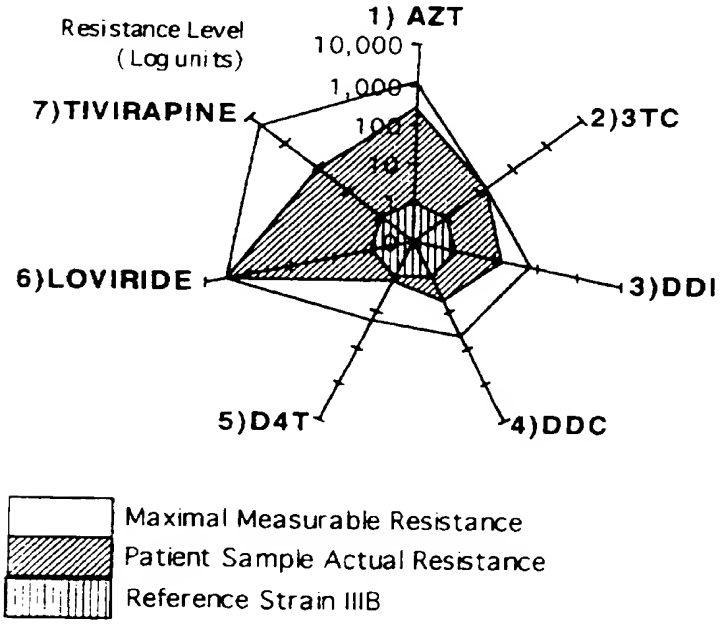
Resistance Level
(Log units)
1) AZT
10,000
1,000
100
10
1
2)3TC
3)DDI
4)DDC
5)D4T
6)LOVIRIDE
7)TIVIRAPINE
Maximal Measurable Resistance
Patient Sample Actual Resistance
Reference Strain IIIB

Fig. 8B

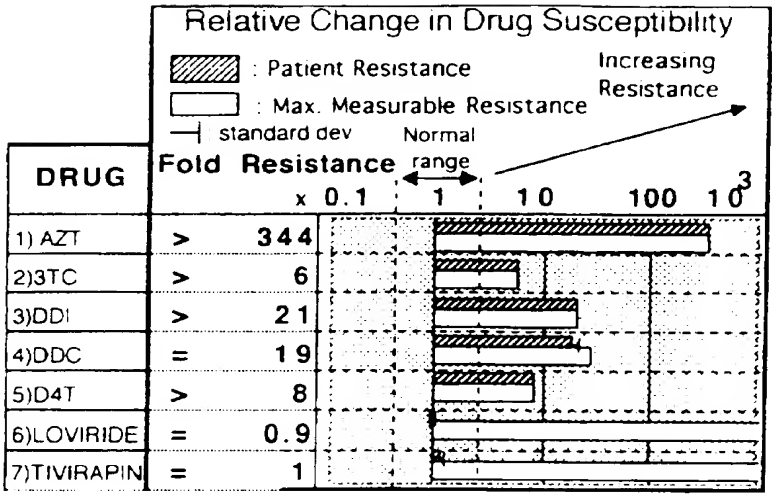
Relative Change in Drug Susceptibility
: Patient Resistance
: Max. Measurable Resistance
standard dev
Normal range
Increasing Resistance
DRUG
Fold Resistance
x 0.1
1
10
100
10 3
1) AZT > 344
2)3TC > 6
3)DDI > 21
4)DDC = 19
5)D4T > 8
6)LOVIRIDE = 0.9
7)TIVIRAPIN = 1

Fig. 9A

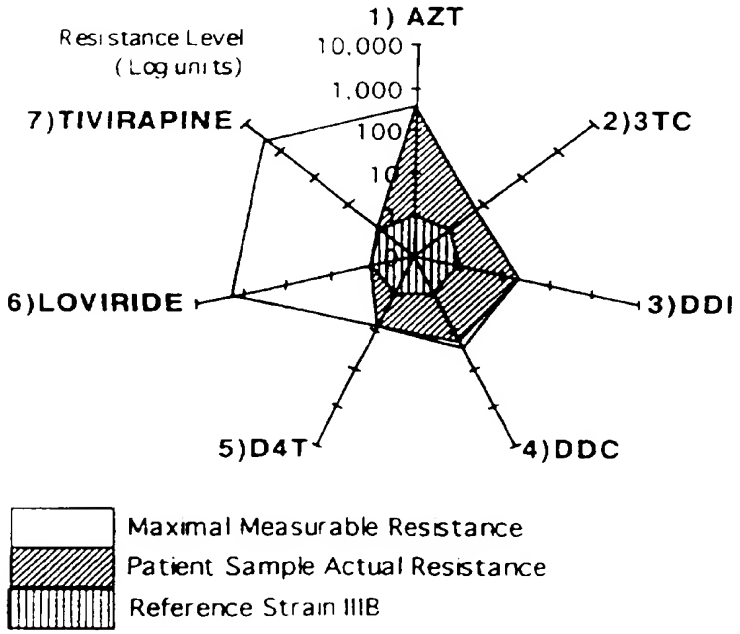
1) AZT
Resistance Level
(Log units)
10,000
1,000
100
10
7)TIVIRAPINE
2)3TC
6)LOVIRIDE
3)DDI
5)D4T
4)DDC
Maximal Measurable Resistance
Patient Sample Actual Resistance
Reference Strain IIIB

Fig. 9B

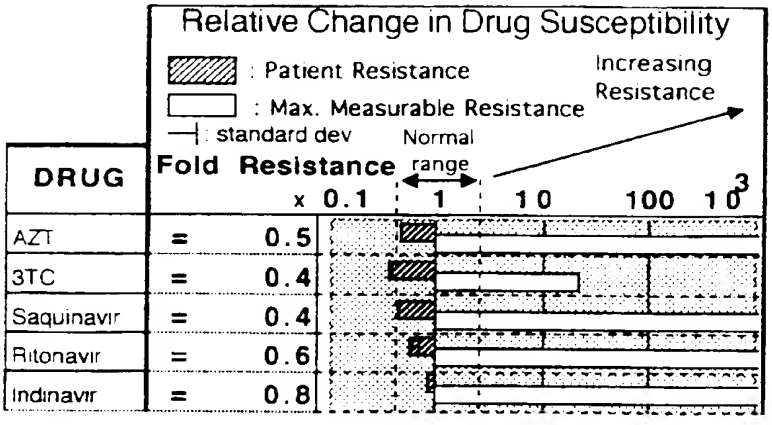

Fig. 10A

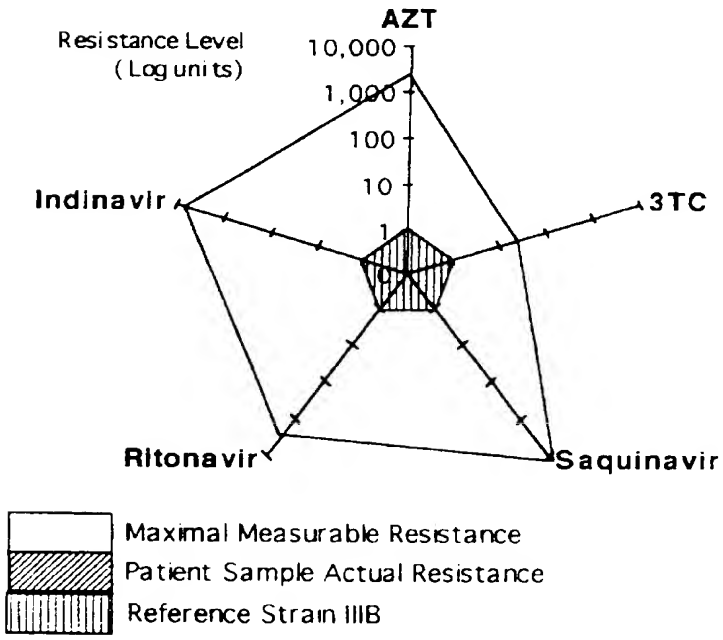

Fig. 10B

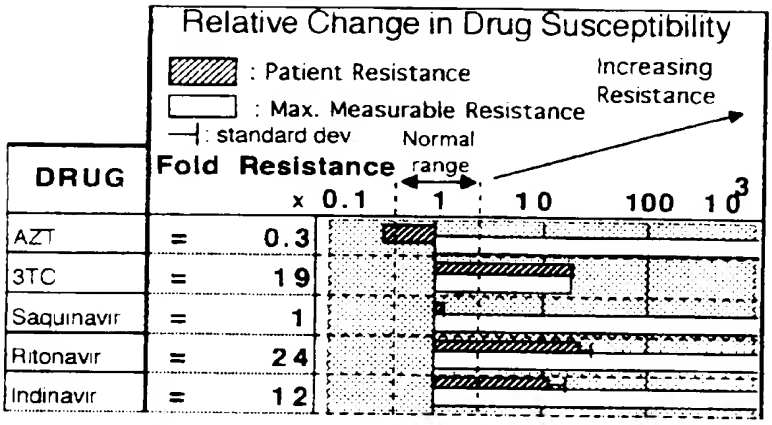

Fig. 11A

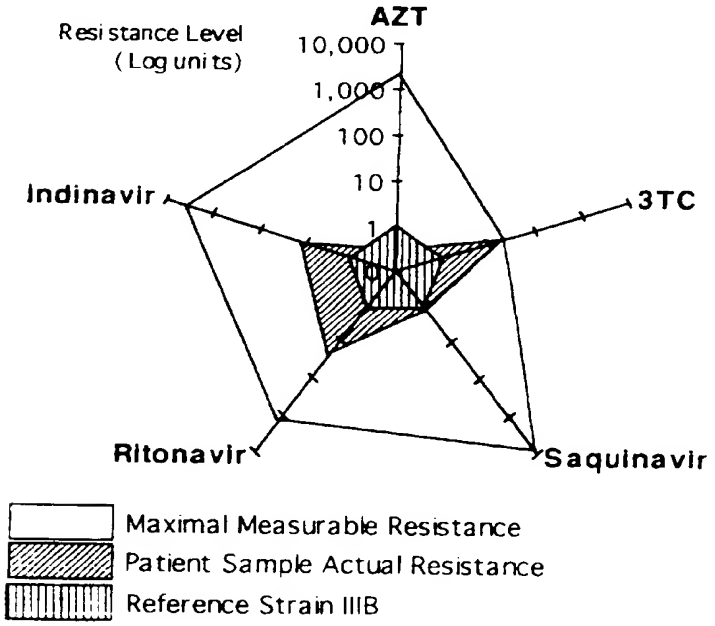

Fig. 11B

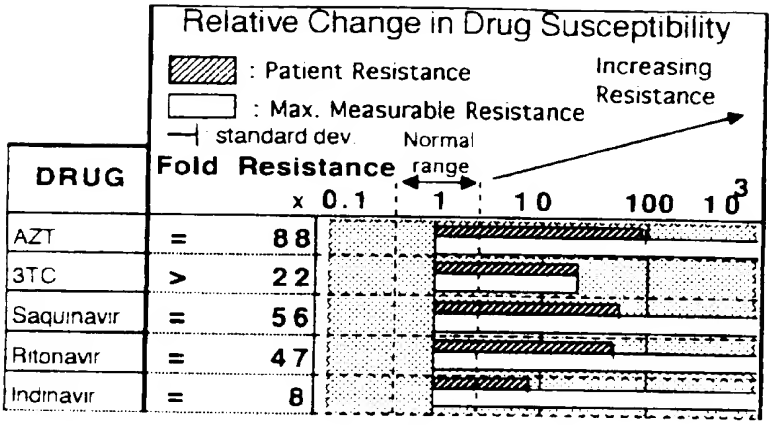

Fig. 12A

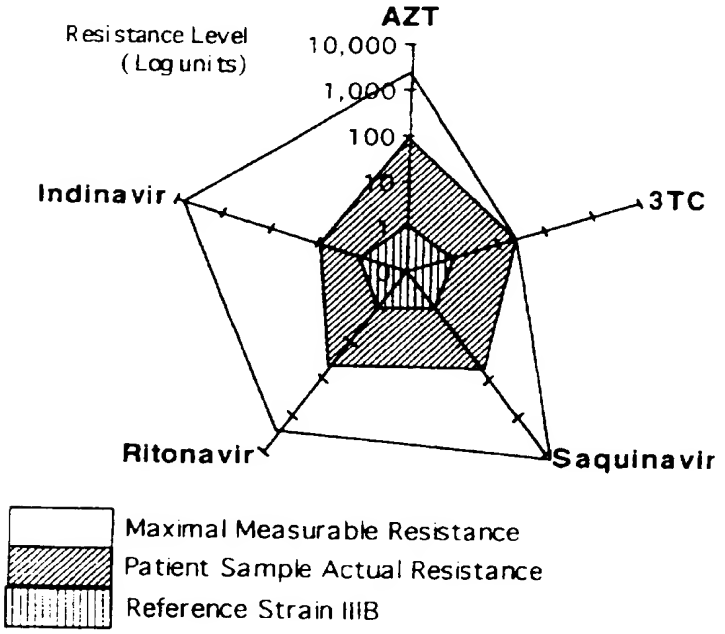

Fig. 12B

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC1/IB 97/00071

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 3 875 396 A (WEBB STEPHEN R) 1 April 1975 see figure 1 ---	7
A,P	WO 96 08580 A (SEPRACOR INC ;MELNICK LAURENCE M (US); HEEFNER DONALD L (US)) 21 March 1996 see the whole document -----	1-29

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 June 1997	24.06.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hoekstra, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter ...nal Application No
PC1/IB 97/00071

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 3875396 A	01-04-75	NONE	
WO 9608580 A	21-03-96	AU 3635295 A	29-03-96
		CA 2199805 A	21-03-96